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Local pharmacological induction of angiogenesis: Drugs for cells and cells as drugs

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ABSTRACT

The past decades have seen significant advances in pro-angiogenic strategies based on delivery of molecules and cells for conditions such as coronary artery disease, critical limb ischemia and stroke. Currently, three major strategies are evolving. Firstly, various pharmacological agents (growth factors, interleukins, small molecules, DNA/RNA) are locally applied at the ischemic region. Secondly, preparations of living cells with considerable bandwidth of tissue origin, differentiation state and preconditioning are delivered locally, rarely systemically. Thirdly, based on the notion, that cellular effects can be attributed mostly to factors secreted *in situ*, the cellular secretome (conditioned media, exosomes) has come into the spotlight. We review these three strategies to achieve (neo) angiogenesis in ischemic tissue with focus on the angiogenic mechanisms they tackle, such as transcription cascades, specific signalling steps and cellular gases. We also include cancer-therapy relevant lymphangiogenesis, and shall seek to explain why there are often conflicting data between *in vitro* and *in vivo*. The lion's share of data encompassing all three approaches comes from experimental animal work and we shall highlight common technical obstacles in the delivery of therapeutic molecules, cells, and secretome. This plethora of preclinical data contrasts with a dearth of clinical studies. A lack of adequate delivery vehicles and standardised assessment of clinical outcomes might play a role here, as well as regulatory, IP, and manufacturing constraints of candidate compounds; in addition, completed clinical trials have yet to reveal a successful and efficacious strategy. As the biology of angiogenesis is understood well enough for clinical purposes, it will be a matter of time to achieve success for well-stratified patients, and most probably with a combination of compounds.

Abbreviations: α-SMA, alpha smooth muscle actin; aFGF, acidic fibroblastic growth factor; ADSCs, adipose derived stem cells; Akt, serine/threonine specific protein kinase; Ang1, angiopoietin 1; FGF-2, basic fibroblastic growth factor; BMSCs, bone marrow mesenchymal stem cells; BM-MNCs, bone marrow mononuclear cells; CABG, coronary artery bypass grafting; CAD, coronary artery disease; CAM, chorioallantoic membrane; CD, cluster of differentiation; CLI, critical limb ischemia; COX-1, cytochrome c oxidase subunit 1; CST, compressive sleeve therapy; EC, endothelial cells; ECM, extracellular matrix; ELF, extremely low frequency; EMF, electromagnetic field stimulation; eNOS, endothelial nitric oxide synthase 3; EPCs, endothelial progenitor cells; ePTFE, polytetrafluoroethylene; ERK, extracellular signal regulated kinases; GAG, glycosaminoglycan; G-CSF, granulocyte -colony stimulating factor; GF, growth factor; hB-EGF, heparin binding endothelial growth factor; HGF, hepatocyte growth factor; HIF, hypoxia inducible factor; HUVEC, human umbilical vein endothelial cells; ICAM, intercellular adhesion molecule; Id1, DNA-binding protein inhibitor; IGF-1, insulin-like growth factor 1; IL, interleukin; ISL1, insulin gene enhancer protein; iNOS, inducible nitric oxide synthase; JNK, c-Jun N-terminal kinases; LIPUS, low-intensity pulsed ultrasounds; LLLT, low-level laser therapy; miRNA, micro ribonucleic acid; MMPs, metalloproteinases; MNCs, mononuclear cells; MVEs, multi-vesicular endosomes; NGF, nerve growth factor; NO, nitric oxide; NOS, nitric oxide synthase; PAD, peripheral artery disease; PDGF, platelet derived growth factor; PEG, polyethylene glycol; PEMF, pulsed electromagnetic field stimulation; PH, prolyl hydroxylase; PI3K, phosphatidylinositol-3-kinases; PLGA, poly(lactic-*co*-glycolic acid); PlGF, placental growth factor; PMA, phorbol 12-myristate 13-acetate; PRF, platelet rich fibrin; Prox-1, prospero homeobox protein 1; PRP, platelet rich plasma; Rac1, ras-related C3 botulinum toxin substrate 1; SDF-1α, stromal cell-derived factor 1 alpha; SMCs, smooth muscle cells; Src, proto-oncogene tyrosine-protein kinase Src; Tie2, angiopoietin 1 receptor; TIMP, tissue inhibitor of metalloproteinase; TGF-β, transforming growth factor beta; uPa, urokinase-type plasminogen activator; VCAM-1, vascular cell adhesion protein; VE-cadherin, vascular endothelial cadherin; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; UC-MSCs, umbilical cord mesenchymal stem cells.

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Contents

1. Introduction

For almost all human tissues, a direct vascular perfusion at the micro level (4–7 μm capillaries, lymphatics), derived from a network of blood vessels at the macro level (1 mm to 2 cm, arterioles, arteries, venules, veins, lymph vessels and ducts), is mandatory to sustain metabolic function for tissue maintenance, growth and repair [1,2]. The continuous flow of blood and its corpuscular elements ensures constant exchange of nutrients, metabolic waste and gases via diffusion through capillary walls [3]. Lymphatic vessels serve as a transport system for fluid and large proteins collected from the interstitial space, immune cells and also lipid droplets after resorption in the intestine [4,5]. Tissues that are not vascularised, such as cartilage and cornea, are dependent on the exchange of nutrients, waste and gases via diffusion from adjacent vascularised tissues [6,7]. Microangiogenesis is a prerequisite for tissue growth. First, in the embryo, angioblasts, progenitor cells for both endothelial cells (EC) and hematopoietic cells, form clusters that are sustained via diffusion of oxygen and nutrients through the uterine wall. In these aggregates, the inner cells will become the hematopoietic precursors while the outer cells will give rise to EC [8]. The endothelial cells start forming a primitive capillary network along which tissue grows forming more complex structures. When tissue size reaches the limit of oxygen diffusion, further vessel growth is prompted through hypoxia and increased expression of hypoxia inducible transcription factors (HIF) (Fig. 1) [9]. The vascular network increasingly expands through sprouting mediated by EC, which later are covered by pericytes and vascular smooth muscle cells (SMCs) to provide strength and stability to the new vessel [8]. In adulthood, EC also proliferate in response to stimuli, (e.g. hypoxia) to initiate angiogenesis [10]. Once EC are activated, vasodilation of the parental vessel occurs, creating space between the EC, which allows them to migrate to the interstitial space and proliferate to form the elongating tip of a new capillary [11]. This process also involves activation of metalloproteinases (MMPs) to degrade the basement membrane also releasing/activating growth factors trapped within the ECM. This is mediated by RhoA/Rho kinase signalling as it involves significant cytoskeletal remodelling [12]. To stabilise the blood vessel, similarly to vasculogenesis, pericytes and SMCs are recruited and ECM is deposited by them. The increasing shear stress from the blood flow will contribute to vessel stability derived from further ECM remodelling and EC – pericytes/smooth muscle cell interactions [13].

As for the formation of lymphatic vessels, lymphangiogenesis in the embryo is only initiated after the vascular system is already established. A distinct subpopulation of EC commits to the lymphatic lineage and sprouts to form primitive structures that constitute the lymph sacs, from which peripheral lymphatic vessels are derived through centrifugal sprouting. Different lymphatic capillary networks eventually connect and undergo further remodelling and maturation [14]. In adulthood, lymphangiogenesis occurs primarily through sprouting from pre-existing vessels during wound healing and development of the corpus luteum. However, growth of lymphatic vessels is also associated with various pathophysiologies including tumour metastasis and inflammation [15,16]. Disorders in the lymphatic system can occur due to malformations, as a consequence of localised trauma and after surgery or radiation therapy for malignancies, particularly of the breast or pelvic organs [17,18]. The impaired lymphatic fluid flow leads to lymphoedema, for which there are currently very limited treatments mostly dependent on physiotherapy [19].

Restriction of blood flow on the macrolevel leads to reduced perfusion of the capillary bed downstream of the feeder vessel. Thus, the affected area becomes underperfused, or ischemic, there is a shortage of oxygen, leading to hypoxia in the tissue, and metabolic waste accumulates [20]. This is characteristic of pathological conditions such as coronary artery disease (CAD), critical limb ischemia (CLI), myocardial infarction or ischemic stroke. Ischemia onset is usually gradual, caused by changes in the vessel walls leading to stenosis, but can progress to dangerous levels (CLI, crescendo angina pectoris) and be suddenly aggravated by a blood clot that either rapidly forms at a wall lesion (thrombosis) and occludes the lumen or has formed there but is

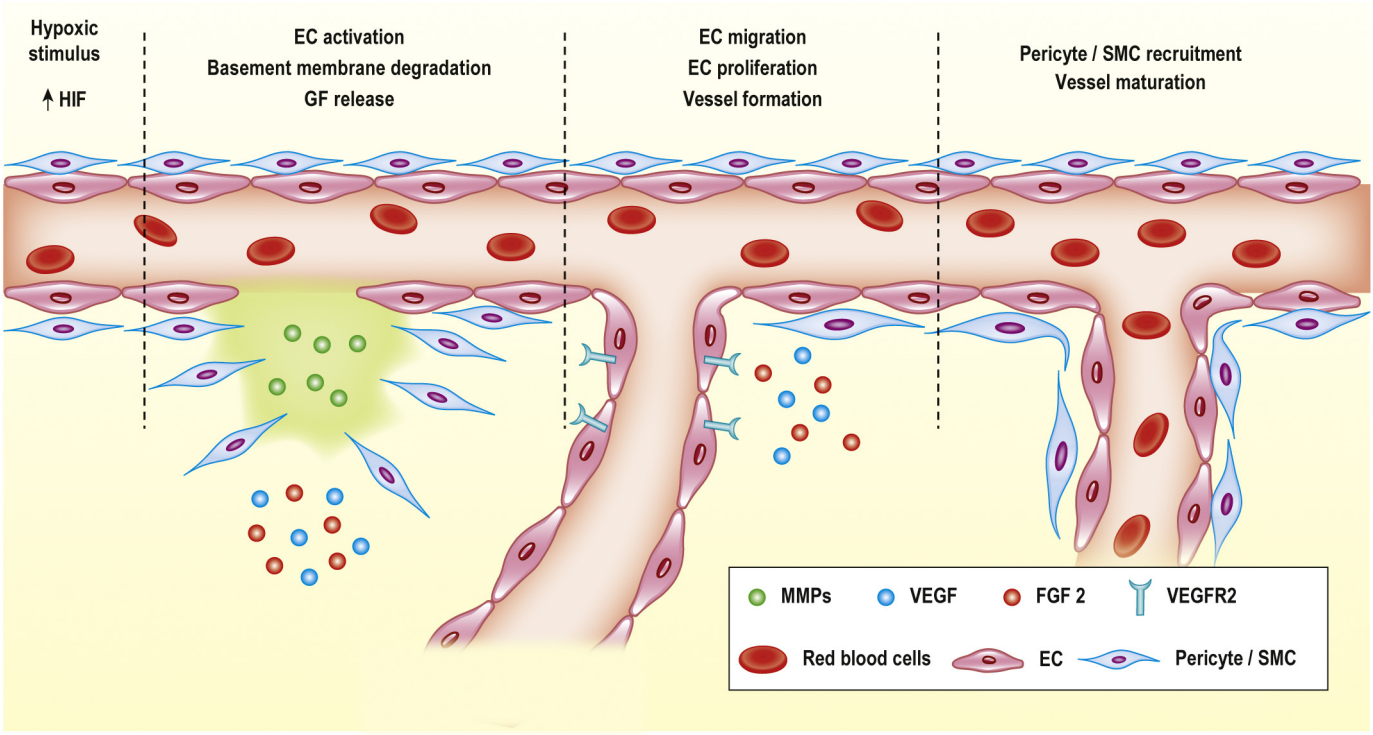

Fig. 1. Overview of the main events of angiogenesis.

transported to another location in the vasculature where it finally occludes a vessel (embolus) [21,22].

On the microlevel, a diffuse obliteration process of small vessels and capillaries can lead to ischemia as well, triggered by metabolic aberrances such as hyperglycaemia in diabetes, but also homocystinuria and inflammatory conditions such as lupus erythematosus [23–25]. Damage of retinal capillaries, nerves and loss of filtration function of renal glomeruli are all manifestations of capillary injury [26–28]. Furthermore, diabetes also causes a macroangiopathy which can be explained by direct damage of vessel wall constituents, but also the obliteration of capillaries nourishing the vessel wall (vasa vasorum) [29,30].

Depending on anatomical locations vascularisation strategies tackling the macro level have to be different from those on the micro level, though in many cases pathologies on both levels occur simultaneously.

Current clinical methods to restore blood flow are mainly focussing on the macro level with the expectation that the micro level is then served by default. In essence, these strategies either seek to bypass or substitute the occlusion, or directly address the stenotic lesion directly by mechanical and pharmacological means [20]. These strategies can be categorised as reperfusion therapies and encompass long term revascularisation, however, their application depends on the blood vessels to be treated or replaced, respectively. Synthetic vascular grafts, such as polytetrafluoroethylene (ePTFE), polyethylene terephthalate (Dacron®) or polyurethane are extensively utilised clinically but are more successful for applications with a vessel diameter larger than 6 mm, with neointimal hyperplasia being the major cause of failure when these are used in smaller calibre applications [31,32]. For coronary bypasses, a homograft is used. The saphenous vein, internal mammary artery, or radial artery are suitable options for small diameter grafts (˜6 mm) [31]. Alternatively to homografts, balloon dilation with subsequent stenting is employed. Complications of stenting are restenosis due to hyperplasia of smooth muscle cells leading to a neointima formation and restenosis [33]. To mitigate this, drug eluting stents and balloons are in clinical use [34,35]. With regards to long term patency, coronary artery bypass grafting (CABG) and stenting are comparable in clinical outcomes. Functionalisation of synthetic grafts with various molecules (e.g. proteins, glycosaminoglycans, growth factors, peptides) has also been attempted [14,36,37], however, clinical studies to date have focussed on larger calibre vessels (e.g. femoropopliteal bypass or aortic reconstruction procedures) [38–42].

An acute perfusion problem on the micro level occurs also when *in vitro* cultured tissue is implanted into living tissue and immediately requires vascularisation to survive. Another current tissue engineering problem for regenerative procedures is that standard culture runs at atmospheric oxygen, meaning 21% oxygen saturation in culture media. Depending on diffusion conditions in the culture vessel and medium, this constitutes hyperoxia. Physiological oxygen levels range from 0.5% to 9% (~1% in the intervertebral disc and certain areas of the brain [43,44], ~6% in bone marrow [45], ~5% in cartilage [46]) and implanting a typically non-vascularised hyperoxic tissue piece into a living tissue will lead to a race between central necrosis of the implant and its vascularisation via capillaries coming from the host tissue; they would be sprouting from a pre-existing capillary network. In summary, strategies on the macro level are primarily to establish reperfusion by surgical means, and to prevent restenosis by mechanical and pharmacological means [47,48]. The obvious, yet challenging, solution lies on promoting vascular perfusion of the implanted constructs or ischemic tissue through angiogenesis, the formation of new capillaries from pre-existing blood vessels. Efforts are increasingly directed towards developing pro-angiogenic therapies that locally stimulate native or implanted cells to revascularise ischemic areas. This can involve the delivery of drugs (e.g. growth factors, cytokines, small molecules) or cells (untreated, pre-conditioned, genetically modified, secretome). Growth factors are the most frequently used category of pro-angiogenic molecules. These include vascular endothelial growth factor (VEGF), fibroblastic growth factor 2 (FGF-2) and hepatocyte growth factor (HGF) - all of which are powerful mitogens – and platelet derived growth factor (PDGF), transforming growth factor beta (TGF-β), angiopoietins (Ang) and placental growth factor 1 (PlGF-1). Other molecules have shown angiogenic potential, as well, for example

interleukins (IL), nitric oxide (NO), copper and calcium ions and phorbol 12-myristate 13-acetate (PMA) [49–52]. Regarding cell delivery, EC are favoured for *in vitro* testing, while progenitor (e.g. bone marrow mononuclear cells [BM-MNCs], EPCs) and stem cells (bone marrow mesenchymal stem cells [BMSCs], adipose derived stem cells [ADSCs]) have been preferentially used *in vivo* and clinically [53,54]. BM-MNCs present a significant advantage, as they do not require expansion in culture, however, similarly to EPCs, they are a heterogeneous, poorly defined population. BMSCs and ADSCs are also appealing sources due to their multipotency and ease of extraction and expansion, however, their implantation has been associated with restenosis and atherogenicity [55]. Therefore, conditioning of cells prior to implantation with hypoxia, mechanical stimulation, or genetic manipulation has been explored with the aim to better control the behaviour of the cells and their secretion profile to overcome such issues [56,57]. As an alternative to whole cell implantation, cell-derived vesicles can be utilised to exploit the cell secretome, since they have been shown to contain both proteins and nucleic acids and to play a crucial role in cell-cell communication [58,59].

Several of these approaches have been extensively assessed *in vitro*, *in vivo*, and clinically. There are well-established two- and three- dimensional *in vitro* models that allow for the assessment of the suitability of a therapeutic agent or a cell source. Two-dimensional models comprise seeding cells on tissue culture plastic coated with adhesive proteins, while three-dimensional models typically use hydrogels (e.g. collagen, fibrin, plasma clot, Matrigel, or mixtures) and are based on the ability of EC to invade the substrate in response to a drug/treatment. Cells can be directly seeded on top of gels, sandwiched between two gel layers, seeded dispersedly, clustered as spheroids or attached to microcarrier beads and scattered throughout the gel [60,61]. The three-dimensional models present the advantage of allowing for representation of the different stages of angiogenesis: sprouting, branching and network formation. *Ex vivo* models based on culture of rat aortic rings and assessment of outgrowths or rat mesentery for study of lymphangiogenesis alongside angiogenesis have also been described [62,63]. More recent *in vitro* models use microfluidic channels fabricated by soft lithography [64]. Other models attempt to imitate the complexity of cell-cell interactions and their paracrine effect by co-culture methods or conditioned media [65,66]. Nonetheless, preclinical assessment currently appears to be the most accurate way of assessing the angiogenic potential of cells or drugs. Most commonly used preclinical animal models include the chick embryo chorioallantoic membrane (CAM) assay, dorsal skinfold chamber, cornea micro-pocket assay, subcutaneous implantation, hindlimb ischemia and myocardial infarction models [67,68].

Current advancements in pro-angiogenic therapies can be grouped into the delivery of drugs and the delivery of cells. Herein, we critically assess the *in vitro*, *in vivo* and clinical progress that has been made in the field of drug and cell delivery, respectively, for the promotion of microvascularisation (Table 1).

2. Drugs for cells

The most common pro-angiogenic therapeutic approach is based on the delivery of therapeutic molecules that can include inorganic compounds, cytokines or growth factors; from the group of the latter

Table 1
Overview of different pharmacological approaches for angiogenesis and current limitations.

	Approaches	Description	Current limitations
Drugs for cells	Growth factors, cytokines	Delivery of growth factors and cytokines, individually or in mixtures that directly (direct stimulation of ECs) or indirectly (e.g. cell recruitment, vessel stabilisation, maturation) promote angiogenesis	Short half-life Poor local retention Dosage High cost Need for delivery vehicles
	Interleukins, ions, gases, small molecules	Delivery of small molecules that promote angiogenic events (e.g. cell migration, cytoskeletal reorganisation, tube formation, ECM stabilisation, interaction with growth factors)	Limited data available (primarily *in vitro*) Short half-life Poor local retention
	Hypoxia mimicking strategies	Delivery of small molecules that promote angiogenesis through stimulation of oxygen-independent activation of hypoxia inducible factor (HIF)	The use of unspecific PH inhibitors can impair normal collagen deposition Lack of clinical testing for angiogenic applications
	Genes	Delivery of genes to cells at injury/diseased site to promote sustained delivery of pro-angiogenic secretome	Localisation Dosage (conducive to secretion of supraphysiological dosages of growth factors) Control of duration of transgene expression Unknown long term adverse effects Regulatory hurdles
	Physical stimuli	Use of external physical stimulation to promote localised increase of pro-angiogenic secretome	Limited data available Further understanding required at cell/molecular level Lack of standardised protocols
Cells as drugs	Naïve cells	Delivery of cells to promote direct localised pro-angiogenic action	Cell availability Poor local retention/cell attachment Poor survival
	Pre-conditioned cells	Delivery of cells that have been exposed to external pre-conditioning (e.g. hypoxia, mechanical and electromagnetic stimulation) prior to implantation to promote angiogenic phenotype and secretome	Cell availability Poor local retention/cell attachment Poor survival Limited data available Highly variable preconditioning protocols
	Genetically modified cells	Delivery of cells that have been genetically modified prior to implantation to modulate secretome	Cell availability Poor local retention/cell attachment Poor survival Limited data available
	Cell secretome and cell-derived vesicles	Delivery of conditioned media or extracellular vesicles. Full secretome versus delivery of a single molecule	Poor local retention Limited cargo characterisation Dosage Limited data available

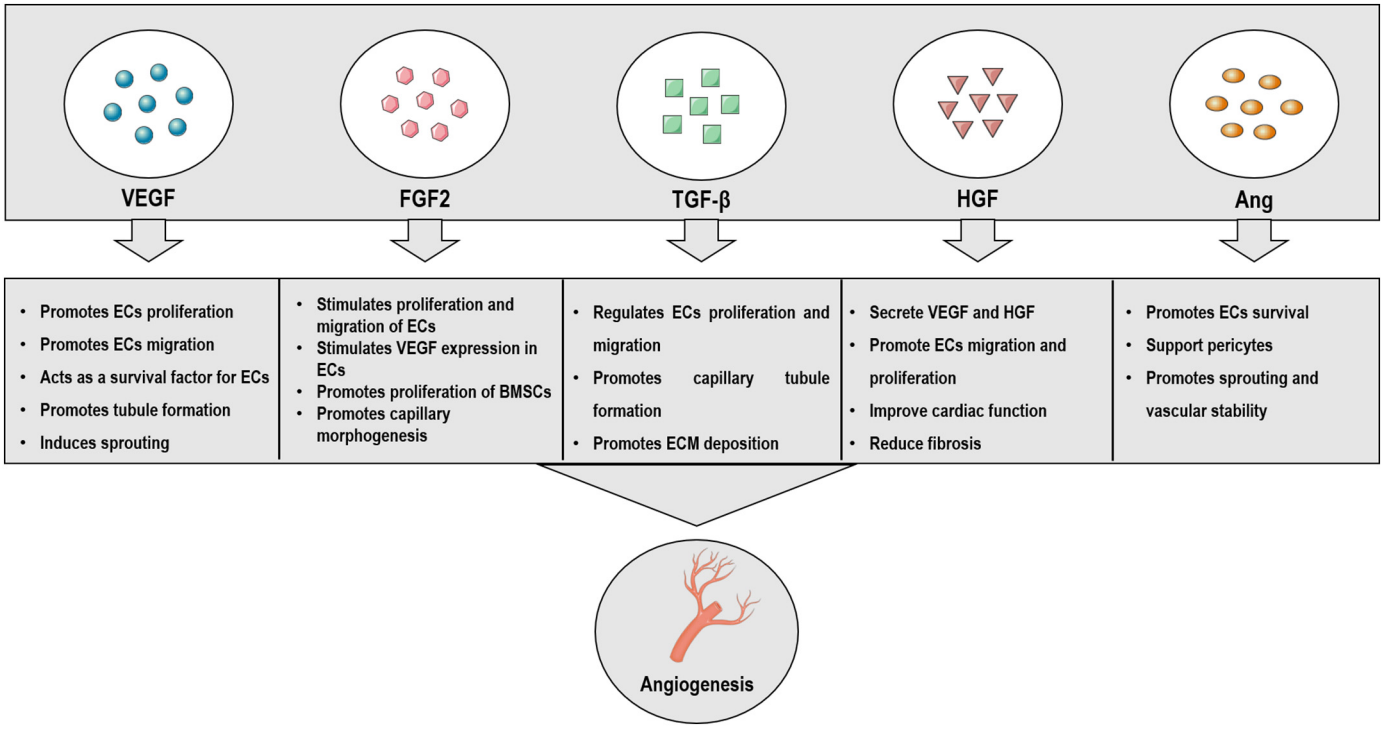

Fig. 2. Most commonly therapeutic targets used in peptide or gene form and main outcomes observed after their delivery.

VEGF, FGF-2, PDGF and HGF are the most frequently used (Fig. 2) [69]. *In vitro* testing of these molecules involves culture media supplementation, while *in vivo* and clinically, these molecules are typically injected directly into the tissue location of interest. Defined mixtures of different therapeutic factors have also been used or, alternatively, platelet rich plasma (PRP) or similar mixtures including various proteins, cytokines and growth factors [70].

Similar to cell delivery, drug injections present challenges regarding half-life and local retention of active molecules [71]. To overcome these issues, sustained delivery using scaffolds (e.g. hydrogels, sponges, fibres, particles, liposomes) has been extensively tested along with binding to other molecules (e.g. peptides, glycosaminoglycans, sulphated molecules) [71,72]. Another widely tested approach, is the delivery of DNA/RNA using vehicles for gene transfer of the cells *in situ*, thereby ensuring a more prolonged and sustained delivery of the molecule of interest. The most commonly delivered genes encode the most frequently used growth factors in peptide form, such as VEGF and FGF-2, though other genes targeting relevant pathways have also been assessed [73–75]. In this section, we will focus on *in vitro*, preclinical and clinical studies that report the use of therapeutic factors to modulate cell behaviour for promotion of angiogenesis, as well as their mode of delivery (e.g. injection, use of vehicle, gene transfer).

2.1. Delivery of growth factors and cytokines

2.1.1. In vitro and preclinical assessment of individual growth factors

The terms “growth factor” and “cytokine” are often interchangeably used. Cytokines are a broad and loose category of small proteins ranging from 5 to 25 kDa, whereas growth factors can generally be seen as a subset that can reach up to 100 kDa. Cytokines and growth factors are produced by a broad range of cells, however, it is often non-endothelial cells that drive endothelial cell behaviour to promote angiogenesis by secreting growth factors or cytokines. The most frequently used growth factor to modulate angiogenesis *in vitro* and *in vivo* is VEGF due to its pivotal role in initiating angiogenesis, and as survival factor for cultured endothelial cells [76]. *In vitro* supplementation with $VEGF_{165}$ promotes EC proliferation, survival and capillary formation in Matrigel, collagen gels and poly(lactic-*co*-glycolic acid) (PLGA)/polyethylene glycol (PEG) microspheres [77–79]. In high doses (50 ng/ml), VEGF appears to initiate endothelial differentiation of BMSCs, as evidenced by typical markers such as von Willebrand factor, and formation of capillary-like structures in a gel matrix [79]. VEGF-mimicking peptides promote endothelial sprouting and tube formation from aortic rings, promote EC proliferation, migration and survival through activation of VEGF receptor 2 (VEGFR2) [80].

Though pharmacologic strategies for lymphangiogenesis are still very much in their infancy when compared with angiogenesis, several targets of interest have been identified [19]. VEGF-C and VEGF-D are known to act on lymphatic EC through VEGFR-3 activation and *in vitro* modulate their proliferation and organisation. In fact, VEGFR-3 and transcription factor prospero homeobox protein 1 (Prox-1) are essential for embryonic lymphatic development [81,82]. Irreversible lymphatic phenotype commitment is characterised by simultaneous expression of not only VEGFR-3 and Prox-1 but also lymphatic vessel endothelial hyaluronan receptor (LYVE-1) and secondary lymphoid organ chemokine (SLC) [19].

Although the key role of VEGF in angiogenesis (EC proliferation and viability) is beyond doubt, it also has become clear that VEGF alone is not sufficient to ensure stable vascular networks *in vitro* [83–85] and *in vivo*. Animal experiments have repeatedly shown that high local tissue VEGF concentrations leads to tortuous and leaky capillaries [86,87].

This has led to consider additional angiogenic factors. FGF-2 is another potent inducer of angiogenesis; it stimulates proliferation, migration and VEGF expression in ECs [88,89]. BMSCs and fibroblasts are strong producers of VEGF and FGF-2, thus targeting EC and inducing capillary sprouting and network formation [90–92]. When stimulated by FGF-2 on the surface of collagen gels, EC produce more urokinase-type plasminogen activator (uPa), a protease involved in vascular permeability and ECM degradation [83,93].

FGF-2 also promotes proliferation of BMSCs [94] and increased capillary tube length in HUVEC/dermal fibroblast co-culture [95].

Several other growth factors contribute significantly to angiogenesis, yet do not act directly on EC. These factors recruit auxiliary cells for vessel stabilisation and maturation: fibroblasts smooth muscle cells (SMCs) and pericytes [96]. This is the case for TGF-β, which inhibits EC proliferation *in vitro* while promoting angiogenesis *in vivo*. However, *in vitro*, when combined with FGF-2 or VEGF, it can have a pro- or anti-angiogenic effect, depending on its concentration [97]. While this highlights the complexity of growth factor interactions during angiogenesis, it also sheds light on the influence of experimental conditions that seem to vary widely across research groups.

Several of the above-mentioned growth factors have been assessed preclinically for their angiogenic potential. VEGF injection or sustained delivery using scaffolds have shown improved capillary density, maturation and neovascularisation in ischemic and subcutaneous models [98–104]. Similar results were obtained with FGF-2 in subcutaneous and ischemic models using delivery systems for implantation [94,105–111]. Furthermore, HGF, although not extensively studied *in vitro*, has also shown increased capillary outgrowth in a rabbit cornea pocket model [112] and improved collateral vessel formation in a rat hindlimb ischemic model, when delivered in repeated doses [113]. Placenta-derived growth factor 1 (PlGF1) has also shown promising results *in vivo*. Implantation in a subcutaneous rat model of a multidomain peptide hydrogel with PlGF1 loaded liposomes led to improved cell infiltration, vessel density and maturation over unloaded hydrogels [114].

Growth factor delivery for therapeutic lymphangiogenesis has also been tested preclinically, though limited data are currently available. Injecting VEGF-C subcutaneously has been shown to improve lymphatic vessel formation and function in a rabbit ear model [115]. Delivery of VEGF-C has also been performed using carriers, such as fibrin, which has led to improved lymphangiogenesis in skin wound healing in a diabetic model [116]. IGF-1, IGF-2 and HGF have also shown increased lymphatic vessel density in mouse cornea assay model [117,118].

2.1.2. In vitro and preclinical assessment of growth factor mixtures

To better mimic spatiotemporal interaction of the various growth factors during angiogenesis and to overcome limitations using single factors, combinations of different molecules have been tested *in vitro*. In some instances, growth factors act synergistically, as is the case with VEGF and FGF-2. On their own, they promote EC invasion of gel matrices and tube formation *in vitro*, but their combination gives a larger than additive effect with a faster angiogenic response and allows the use of lower concentrations of both growth factors [119–123]. Co-delivery of VEGF and HGF in a heparin coacervate increased HUVEC proliferation as well as tube formation in a fibrin gel, presenting improved results over the individual growth factors [124]. Combined delivery of VEGF and heparin stabilised PDGF-BB in a fibrin gel, increased EC proliferation and promoted vessel outgrowth in an aortic ring assay [125]. Systems of increasing complexity can be found in the literature as well, including cocktails of more than two molecules or controlled temporal delivery of multiple molecules [126]. An alternative to well-defined combinations of growth factors is the use of platelet rich plasma (PRP), as it contains a mixture of important angiogenic factors such as VEGF, PDGF-BB, FGF-2, angiopoietins and TGF-β [127,128]. *In vitro*, both PRP and platelet rich fibrin (PRF), which also releases platelet derived factors, can promote EC proliferation and migration [129], likely derived from activation of ERK and serine/threonine specific protein kinase (Akt) pathway [70] and angiopoietin 1 (Ang1) – angiopoietin 1 receptor (Tie2) signalling [127].

Mixtures of bioactive molecules have also been tested in various animal models. For instance, VEGF, HGF and FGF-2 have been implanted subcutaneously in rats and induced neo-capillary formation and granulation tissue formation [130]. Simultaneous delivery of VEGF and PDGF showed improved recovery of tissue in a rat myocardial infarction model over delivery of VEGF alone [131]. PDG-BB has been used in combination with both VEGF and FGF-2 in a murine Matrigel plug angiogenesis assay and myocardial infarction models and evidenced improved vessel maturation through alpha-smooth muscle actin (α-SMA) staining, highlighting the synergistic effect of these two growth factors during later stages of angiogenesis [125,132]. Due to the intricate spatiotemporal action of growth factors during angiogenesis some studies focus on systems allowing delivery of more than two molecules. VEGF, PDGF-BB and TGF-β1 have been delivered in a sequential manner from alginate sulphate/alginate sponges based on different binding constants of the individual factors, and shown improved results over individual growth factor use [133]. PRP and PRF have also been assessed *in vivo* and shown to increase vessel formation, maturation and ECM deposition in a CAM assay [129].

The use of combinations of different growth factors, though logical when considering the complexity of biological processes, poses significant issues in terms of *in vitro* and preclinical evaluation. The combinations of molecules, concentrations and their simultaneous or sequential delivery are endless and require a well-defined systematic approach for their optimisation. However, with high throughput systems becoming more widely available, such alternatives become more feasible.

2.1.3. Clinical assessment

Despite extensive *in vitro* and preclinical testing of angiogenic proteins as monosubstances or in combination, their efficacy has been tested only in a few clinical trials (Table 2), mainly featuring VEGF [134]. Patients with CAD received intracoronary and intravenous injections of different VEGF doses. Significant improvements were observed with regards to exercise time and angina symptoms only with higher doses [135]. A few studies have assessed safety of FGF-2 injection in humans [136]. The first studies investigated the efficacy of FGF-2 for treatment of peripheral arterial disease [137,138], showing encouraging results in terms of improved walking time. However, in patients with CAD, reduction of angina symptoms were observed, although improvements were not statistically significant at clinical end point due to continued recovery in the placebo group, even though no other treatment had been applied simultaneously [139,140]. Heparin/alginate microcapsules have been used to deliver FGF-2 in patients receiving surgery for coronary bypass, where a dose dependent effect was observed only with higher concentrations, increasing blood perfusion in the target area [141,142]. The sustained delivery appears to promote long-lasting effects [143]. Likewise, FGF1 has also been shown to promote sprouting of a new capillary network at injection site in patients with coronary heart disease [144].

Two clinical trials are currently registered on www.clinicaltrials.gov that assess intracoronary delivery of growth factors for coronary artery disease. In one, VEGF is injected alongside oral supplementation of L-arginine (phase I), the other study is based on FGF1 injection (phase II). Of note, human recombinant PDGF-BB has been FDA/EMEA approved for clinical use since 1997 as topical gel (becaplermin) for chronic wound repair due to its chemotactic and mitogenic effects in angiogenesis [145]. However, an increased neoplasm rate at the site of application has been reported in patients that have been exposed locally to more than three tubes of the gel over the treatment period. [146–148]. Becaplermin gel 0.01% is distributed in a 15 g tube, and current recommendations are to recalculate the weekly dosage. This, however, is dependent on the ulcer size, therefore incomplete healing after 20 weeks should lead to considering the risks of continuing Becaplermin gel [149].

The plethora of preclinical data on local delivery of angiogenic proteins is contrasted by a dearth of clinical data. The reasons for that might be that growth factors are more expensive to manufacture and to store, and have a very short half-life after tissue delivery [150,151]. In fact, the rapid decay of the protein factors once applied to a wound area or injected deeper into tissue might be the reason that these factors are all applied in supraphysiological doses. As discussed above, safety concerns have emerged post marketing for PDGF. From the

Table 2
Clinical studies describing growth factor delivery.

Phase	Growth factor	Delivery method	Condition	Outcome	Reference
Phase I	FGF-2	Intra-arterial injection	Atherosclerotic peripheral arterial disease	Improved blood flow in ischemic leg	[137]
Phase I	FGF-2	Intracoronary injection	Angina pectoris	No symptomatic improvements	[140]
Phase II	FGF-2	Intra-arterial injection	Myocardial and limb ischemia	Improved walking time	[138]
Phase I	FGF-2	Epicardial implantation of heparin/alginate capsules	Coronary artery disease	Reduced angina, improved blood perfusion.	[143]
Phase I	FGF-2	Intracoronary injection	Coronary heart disease	No significant long term symptomatic improvements.	[139]
Phase I	FGF-2	Intracoronary or intravenous injection	Coronary heart disease	Improved resting perfusion.	[445]
Phase I/II	VEGF2	Intracoronary and intravenous injections	Exertional angina	Reduced angina with high dose VEGF at 120 days post-treatment.	[135]
Phase I	VEGF	Intracoronary injection	Coronary artery disease	Trend for improved blood perfusion a high dose VEGF	[446]
Phase I	FGF-1	Intracoronary injection	Chronic myocardial ischemia	Improved vascularisation	[144]
Phase I	GSK1278863	Oral	Peripheral artery disease	No symptomatic or performance benefit ensued	[219]
Phase II	GSK1278863	Oral	Chronic kidney disease	GSK1278863 produced dose-dependent effects on haemoglobin; no differences in VEGF levels	[447,448]
Phase II	FG-4592	Oral	Chronic kidney disease	Increased haemoglobin levels	[218,449]
Phase II	AKB-6548	Oral	Chronic kidney disease	Increase haemoglobin levels and enhanced iron mobilization	[450]

physiological point of view, it makes substantial sense to use different GF in combination to achieve angiogenic synergy, thus allowing lowering the concentration of each single GF locally required. The half-life issue of the GF in the host tissue can certainly be addressed by sulphated sugar moieties or manipulating amino acid sequences in the recombinant proteins. For example, dextran sulphate has been demonstrated to protect FGF-2 from heat and acid inactivation and from proteolytic degradation [152]. Similarly, heparan sulphate sugars have been shown to exhibit excellent selectivity to growth factors and to prolong their bioavailability [153,154]. However, they currently have to be purified from biological material (usually porcine gut) and therefore afford costly purification and manufacturing steps. Be it these stabilising factors, or sustained delivery materials, they add significantly to costs in terms of regulatory hurdles (multiple substances necessary and safety studies), and navigating intellectual property issues.

2.2. Delivery of interleukins, ions, gases, and small molecules

Several IL appear to possess pro-angiogenic potential. While IL-17 appears to promote migration, cytoskeletal reorganisation and capillary formation through upregulation of Rac1 and RhoA, via the phosphatidylinositol 3-kinase (PI3K) signalling pathway [155], IL-18 does so by proto-oncogene tyrosine protein kinase (Src) and JNK signalling [156]. On Matrigel, both IL-2 and IL-6 increase tube formation by HUVEC (increase of VEGF and PDGF) and circulating EPCs, respectively [49,157].

Other factors supporting angiogenesis are metals, earth alkaline metals [158–160] and gases. Indeed, copper is necessary for activation of EC and angiogenic growth factors, including VEGF and FGF-2 [161]. It promotes EC proliferation, even in the absence of serum [51] and increases VEGF secretion in keratinocyte culture [162]. In an aortic ring culture, copper promoted angiogenesis in a concentration dependent manner, with high concentrations suppressing angiogenesis but showing increased VEGF production [163]. The proangiogenic effect of copper has been reported *in vivo* using copper nanoparticles which promoted development of a dense vascular network in a chick embryo [164] and in a subcutaneous implant in mice particularly when used alongside FGF-2 [165]. Nonetheless, the mechanism by which copper influences angiogenesis is still controversial. Of note, copper is an essential cofactor for the copper-dependent amino oxidases. Lysyl oxidase (LOX) stabilises collagen and elastin by paving the path for covalent crosslinks, pyridinolines and desmosines, respectively [166–168]. Interestingly, of the three currently known lysyl oxidase homologs (LOX-like enzymes, LOXL), LOXL2 can also crosslink collagen type IV and hence influence the sprouting of new blood vessels [169]. Further, copper has also been implicated in stabilisation of HIF-1α through prolyl hydroxylase inhibition, which could also explain its role in the early phases of angiogenesis [170]. Despite the interesting results and the use of copper chelators for inhibition of angiogenic response in tumours, further work is required to fully elucidate the pro-angiogenic role of copper [171].

Although not widely assessed *in vitro*, earth alkali metals appear to play a role in angiogenesis. Calcium ions, released by degradation from a bioglass scaffold, have also shown angiogenic potential when implanted subcutaneously on rats [172]. Additionally, several publications have recognised the potential of calcium, magnesium and silicate in vascularisation for regeneration of osteogenic tissue [172–174].

Two other unlikely choices for promotion of angiogenesis are nicotine and sodium arsenite. In well-defined low concentration ranges both have been shown to accelerate capillary tube formation in EC culture, with nicotine acting through nicotinic cholinergic receptors and sodium arsenite through VEGF and NO activity [175–178]. Interestingly, nicotine has been evaluated *in vivo* alongside FGF-2 in a wound healing model where it appeared to reduce wound area and improve capillary formation [179].

PMA, although also reported as a tumour promoter, can induce formation of capillaries in EC culture and increase expression of angiogenic markers, such as CD31 and VE-cadherin [52,84,93]. Its angiogenic activity is likely derived from triggering cell invasiveness through increased production of collagenase and uPa [180].

Cell signalling by gases has gained wide-spread attention with regards to angiogenesis. Nitric oxide (NO) is produced by nitric oxide synthases from L-arginine and plays a key role in angiogenesis. When NO synthase (NOS) activity is reduced, VEGF-induced angiogenesis is hindered [181,182]. NO - stimulating substances indirectly promote EC migration, proliferation and vascular permeability [50,183,184]. Upregulation of eNOS and consequent release of NO in EC is heavily dependent on different growth factors such as VEGF, heparin binding EGF (HB-EGF) and TGF-β1, and on PI3K signalling and integrin $\alpha5\beta3$ activity [185–189]. However, there is controversy regarding the role of NO in angiogenesis, as exogenous supplementation of HUVEC cultures with organic NO donors, presented decreased cell migration [190]. Furthermore, there is *in vivo* data available that appears to substantiate the

anti-angiogenic effect of NO, since it has been demonstrated that the use of NO donors in a CAM model led to decreased collagen synthesis and vascular density [182,191]. In addition, increasing the NO metabolite concentration inhibits EC proliferation [192], indicating the concentration of NO present on the intracellular compartment may dictate the effect it has on angiogenesis.

Hydrogen sulphide (H_2S) has also been known to stimulate angiogenesis. The administration of H_2S to EC culture simulates cell proliferation, migration and tube formation [193]. Exogenous H_2S has been shown increased collateral vessel growth, capillary density, and regional tissue blood flow in a hindlimb ischaemia model [194]. Additionally, in an *in vivo* model of mice Matrigel plug, exogenously administered H_2S significantly promoted neovascularization [195]. However, H_2S does not exhibit a pro-angiogenic effect at a high concentration and it might evoke mechanisms that counteract the pro-angiogenic pathways.

2.2.1. Hypoxia mimicking strategies

Finally, a group of small molecules modulating angiogenesis deserves particular attention due to their well characterised mechanism and their potential clinical value. These are the group of prolyl hydroxylase inhibitors which offer a pharmacological approach to stimulate angiogenesis through oxygen-independent activation of hypoxia inducible factor (HIF). Under normoxia, HIF-prolyl-4-hydroxylase (PH), which is Fe(II) and 2-oxoglutarate dependent, catalyses the hydroxylation of prolines which target HIF subunits for degradation. PH inhibitors therefore stabilise HIF in the absence of hypoxia. Various molecules including pyridine-2, 4-dicarboxylate, 5-oxaproline, alpha alpha-dipyridyl, or hydralazine have been utilised, but others broadly grouped as 2-oxoglutarate analogues or iron chelators have also been assessed [196–200].

As a transcription factor modulating a number of hypoxia response genes, HIF-1α stabilisation will generate not only one, but a variety of angiogenic gene products. These range from VEGF, Ang-1 to erythropoietin, while preparing cells to deal with hypoxia inducing glycolysis [201]. Thus, activating this angiogenic master switch is akin to the external delivery of a mixture of angiogenic factors, as discussed earlier.

In vitro, PH inhibitor treatment of BMSCs have been shown to increase VEGF expression and promote capillary sprouting [202]. *In vivo*, promising results have been obtained in terms of increased vascularisation and bone formation, improved neovascularisation in oxygen-induced retinopathy in a mouse model, protection from ischemia cerebral lesions in a gerbil model, induction of ectopic angiogenesis in zebrafish and enhanced wound healing in diabetic mice [87,202–205]. PHs are a isoenzyme family, of which some members are also essential for thermostability of intracellular collagen triple helices and thus control collagen secretion [206]. Concerns might arise that a unspecific PH inhibitor impairs normal ECM deposition. Therefore, selectivity or preference of PH-inhibitors for HIF-PH or collagen PH in terms of dissociation constants for these iso-enzymes has to be considered, as well as the differential requirement of molecular oxygen of these enzymes [207,208]. However, lack of collagen deposition and thus impaired wound healing has not been an issue so far in preclinical and clinical trials. On the contrary, an antifibrotic effect exerted by broad PH inhibitor - as so-called dirty tool - might add additional benefit to the angiogenic feature [87,196,209]. Importantly, compounds such as hydralazine and ciclopirox were originally developed and approved for different clinical indications (arterial hypertension and skin mycosis, respectively) and have been on the market since 1953 and 1997, respectively. Interestingly, the antifungal drug ciclopirox was first shown to act as PH inhibitor in 2002 [210] and suggested to be anti-angiogenic. Similar predictions were made towards other PH inhibitors in this publication, suggesting them as anti-cancer drugs. Of note, this work was done in HUVEC monocultures in Matrigel angiogenesis assays and chick aortic arch ring sprouting assay [210]. Shortly thereafter, it was shown that in context with mesenchymal cells and unwounded or wounded tissue ciclopirox even as dirty tool, exerts primarily HIF1-alpha stabilising and therefore angiogenic effects [211]. This was corroborated in greater detail in microfluidics systems [209,212] and diabetic wounds in experimental animal models [213,214]. The change viewpoints on PH inhibitors over time in the literature shows the importance of considering and implementing cellular cross-talk in angiogenic assays *in vitro*, as ECs are not the greatest producers of angiogenic factors, but rather the recipients of signals through them. This might explain some of the controversial findings on angiogenic factors within *in vitro* settings, but also in comparison of *in vitro* data with those obtained in animal models. From the pharmacological point of view, PH inhibitors are currently the group of small molecules that progressed farthest and that have been employed in clinical trials.

Clinical data has shown promising results in patients with anaemia derived from chronic kidney disease. PH inhibitors emerge as important alternatives to recombinant erythropoietin, which, in high doses, has been associated with adverse cardiovascular effects [215]. Patients with anaemia derived from end-stage renal disease/chronic kidney disease have shown increased plasma erythropoietin and hemoglobulin levels after oral treatment with PH inhibitors [216–218].

In peripheral artery disease patients, oral treatment with PH inhibitors failed to induce significant changes in terms of exercise performance and mRNA and protein levels of HIF-1α [219]. It was proposed that the results were due to poor tissue penetration of the drug and other localised delivery methods should be considered for such conditions.

2.3. Delivery of genes

2.3.1. In vitro and preclinical assessment

Issues associated with local tissue retention, tissue half-life of growth factors, and manufacturing costs for human recombinant proteins have fuelled interest into delivering genes rather than proteins. Genetic transfer can facilitate overexpression of a molecule of interest in a localised manner for a longer period, similar to sustained delivery through an engineered construct. Gene delivery for pro-angiogenic purposes has been performed using viral (adenovirus, retrovirus) and non-viral vectors (plasmids, liposomes, oligonucleotides, peptides) [220]. Scaffolds have also been used as carriers to try and promote localisation of delivery vehicles and sustained transfer [221]. In most cases, the targeted genes encode the therapeutic molecules that were used in peptide form. *In vitro*, $VEGF_{164}$ overexpression has been promoted in EPCs, through adenoviral transfer, and has increased cell proliferation, adhesion and incorporation into tube-forming HUVEC monolayers [222]. A popular target for genetic manipulation is the NO signalling pathway. Non-viral delivery of eNOS to BMSCs promoted endothelial differentiation through VEGF/PDGFR and FGF-2/FGF-2R signalling pathways, resulting in upregulation of CD31, VEGF and FGF-2 [223]. Transfer of iNOS into EC can also inhibit apoptosis [224]. However, NOS delivered to SMCs and EC has shown reduced proliferation and tube formation *in vitro* [225], and eNOS transfected into EPCS from patients with CAD impaired tube formation in Matrigel, but did promote migration and proliferation under VEGF stimulation [226]. As discussed above, these partially contradictory results might largely reflect the absence of mesenchymal cells in the *in vitro* settings, and therefore assays including additional non-endothelial cells might lead to more consistent data across the board.

Some *in vitro* studies have assessed safety of gene delivery; interestingly, the majority of the work has been performed preclinically and clinically.

Preclinical delivery has focussed on a limited selection of the same therapeutic targets in mice, rat, rabbit, porcine and canine models of wound healing, myocardial infarction and hindlimb ischemia. Intramuscular injections of a VEGF-D gene in rodent and rabbit models have shown increased vessel density and blood perfusion [227]. Additionally, injectable fibrin matrices have also been shown to facilitate intramuscular delivery of the $VEGF_{164}$ gene to ischemic limbs, where a dose-dependent effect was observed, with higher doses leading to formation

of tortuous vessels with large and heterogeneous sizes [228]. Interestingly, sustained delivery was achieved here by incorporating aprotinin into the scaffold to inhibit degradation. Similar observations have been made with inducing local overexpression of VEGF; unregulated VEGF expression has been associated with haemangioma formation and fatal vascular leakage and it has been proposed that these effects are dose-dependent [229–231]. Several studies have pointed out that excessive cell proliferation, hyperplasia, vessel leakage and malformation [230,232–235] are a typical result of supraphysiological local concentrations of VEGF. However, VEGF-induced angioma formation might not appear associated only with dose but with species-dependent VEGF receptor activation, since high doses of mouse VEGF induced angiomas in mice while human VEGF led to normal capillary formation [236]. This finding raises a note of caution on the transferability of animal tests to predict human clinical outcome.

In addition to VEGF, the transfer of several other genes has shown promising results *in vivo*. In hindlimb ischemic models, intramuscular injection of a plasmid bearing the SFD-1α gene, induced significant blood flow recovery, expression of CD31 and mobilisation of EPCs - effects mediated by VEGF regulation, eNOS and Akt signalling pathways [237]. Using a similar model, intramuscular delivery of secretoneurin also contributed to reduced necrosis, upregulation of FGF-2 and PDGF and vessel maturation, assessed by the presence of CD31 and α-SMA. It was observed that these results were highly dependent on NO signalling, since secretoneurin induces activation of eNOS [238]. Regarding myocardial infarction and heart ischemia, viral delivery of IGF-1 has shown improved remodelling mediated by activation of α5 integrins [239]; transfection of HGF can also promote functional recovery after intramuscular delivery [240]; Ang1 viral delivery also facilitated regression of hypertrophic tissue, though it did not affect cardiac function [241]. Remarkably, both HGF and Ang1 genes have been delivered using ultrasound mediated microbubble disruption. This technology employs gas-filled microbubbles that can be loaded with drugs or genes. The microbubbles oscillate when ultrasound waves are applied within their resonance frequency and if higher frequencies are applied, the bubbles burst and release their cargo. This allows for targeted delivery in the area of ultrasound impact, and mild surrounding tissue damage as it can cause permeabilization of neighbouring cell membranes. This delivery method has been used for targeted Ang1 delivery to ECs in ischemic regions overexpressing ICAM-1 after intravenous injection in rabbits after myocardial infarction [242]. ICAM-1 targeted microbubbles adhered to the infarcted area and improved left ventricular function and myocardial perfusion in a comparable manner to Ang1 gene delivery through intramyocardial injection.

Acidic FGF (aFGF), although less utilised in the protein form and not extensively tested *in vitro*, has also been used for therapeutic recovery of myocardial infarction through viral vectors [243]. However, unlike most preclinical studies, the delivery was performed two weeks before induction of myocardial infarction as a preventive treatment. However, results from such studies are difficult to translate as they hardly reflect the clinical reality.

Similar approaches have been applied to gene delivery for therapeutic lymphangiogenesis at a preclinical stage. VEGF-C transduction improved lymphatic network repair in an epigastric mouse model [244], while VEGFR-3 transfer enhanced lymphatic drainage in a lymphoedema skin mice model without collateral effects on the vascular system [245]. Interestingly, VEGF-C localised transduction has also led to improved lymph drainage in an inflammatory arthritis mice model [246]. Transfection with VEGF-C was also shown to improve lymphatic function in rabbit ear and mouse tail lymphoedema models [247]. Furthermore, HGF gene transfer decreased lymphoedema thickness in a rat tail model [248].

Although particular interest has developed around VEGF-C, advantages for its use are still unclear, given that it has also been associated with lymphatic hyperplasia and side effects with regards to blood vessel growth and leakiness [249,250]. These findings appear somewhat similar to the blood microvessel abnormalities produced with locally administered VEGF-A described above. It appears plausible that also in lymphangiogenesis VEGF should not be administered locally as the only factor, but in combination with other agents to balance its effects. Considering the limited data available for *in vitro* and *in vivo* studies, it is no surprise that these approaches are yet to be translated to a clinical setting.

2.3.2. Clinical assessment

In contrast to the previous sections where we pointed out that the clinical data on drug and cell delivery are somehow limited, there are extensive clinical trials on angiogenic gene delivery in humans (Table 3). Several phase I and II clinical trials have assessed mostly plasmid and viral delivery of genes including VEGF, HGF, FGF1, FGF-4, with the majority focussing on VEGF.

For the treatment of chronic CAD, different VEGF splice variants (e.g. $VEGF_{121}$, $VEGF_{165}$) have been utilised. It has been proposed that VEGF gene transfer therapeutic effect derives from improved EPCs recruitment and increased expression of markers such as VEGFR2, CD34, $\alpha5\beta3$ integrin and E-selectin [251]. Intramyocardial injection of adenovirus containing $VEGF_{121}$ has been reported to improve angina symptoms and exercise time however, without benefiting blood perfusion to ischemic areas [252–254]. $VEGF_{165}$ has been the most used in plasmid-mediated delivery, through intramyocardial or percutaneous route and results have been mixed. While several trials report improvements in angina and/or blood perfusion and collateral vessel formation [255–257], others failed to report statistically significant improvements [254,258]. Adenoviral delivery of FGF-4 did not lead to significant improvements in patients with CAD [259]. As for CLI, most investigations have also focussed on $VEGF_{165}$ delivery through intramuscular injection into the affected limb. Adenoviral transfer of $VEGF_{121}$ has failed to produce significant improvements [260] whilst the use of plasmids with $VEGF_{165}$ have shown mixed results with regards to ulcer healing, rest pain, walking time, collateral vessel formation and limb survival [261–263]. Other less studied genes include FGF1 and HGF. Intramuscular plasmid mediated delivery of the FGF1 gene has shown improvements in ulcer healing and limb survival in a phase I study [264], whilst a phase III trial has failed to improve limb survival [265]. Overexpression of HGF has also shown decreased rest pain and increased walking time [266–268]. Furthermore, adenoviral delivery of hypoxia inducible factor 1 alpha (HIF-1α) has failed to show therapeutic effects in ischemic limbs. Although it appeared to lead to a trend in improved ulcer healing, there was a higher amputation rate in the treatment group when compared to placebo [269].

Delivery of genes presents advantages over growth factors as it allows for a continued therapeutic effect. However, despite extensive assessments in both preclinical and clinical settings, a clear benefit in delivering a particular gene has not evolved yet, and further investigations are required to ensure safety of the transferred genes and vehicles.

2.4. Physical stimuli for local secretion of angiogenic factors

Among the strategies to promote angiogenesis, external physical stimulation gained interest due to its non-invasiveness, the ease of use and promising preclinical data. Various types of stimuli have been tested with the rationale that localised physical stimulation can influence the angiogenic secretome, therefore becoming an important alternative to delivery of therapeutic molecules.

Ultrasound is clinically used for therapeutic applications, including tumour ablation, thrombolysis, bone regeneration, and facilitated drug delivery [9]. Therapeutic angiogenic effects of low-intensity ultrasound are hypothesised to stimulate secretion of angiogenic factors, such as VEGF and FGF-2 [270]. Ultrasound irradiation significantly improved limb perfusion and increased blood vessels formation and cell proliferation in a rat model of hindlimb ischemia. Moreover, VEGF mRNA was significantly higher in moderate ischemia [271]. It has

Table 3
Clinical studies describing gene delivery.

Phase	Gene	Delivery method	Condition	Outcome	Reference
Phase I	$VEGF_{121}$	Adenovirus, intramyocardial	Coronary artery disease	Improvement in angina and treadmill exercise time	[252,253]
Phase II	$VEGF_{121}$	Adenovirus, intramyocardial	Chronic myocardial ischemia	Improved angina and exercise time. No significant difference in perfusion	[451]
Phase I	$VEGF_{165}$	Plasmid, intramyocardial	Chronic myocardial ischemia	Improvement in angina and myocardial perfusion	[255]
Phase I	$VEGF_{165}$	Plasmid, intramyocardial	Coronary artery disease	No difference in perfusion though ischemic area reduced significantly in all groups	[254]
Phase I	$VEGF_{165}$	Plasmid, intramyocardial	Angina pectoris	Improved angina, reduce nitroglycerin intake, improved ischemia, improved collateral perfusion	[256]
Phase II	$VEGF_{165}$	Plasmid, percutaneous and intramyocardial	Angina pectoris	Angina improved without significance, no differences in perfusion	[258]
Phase I/II	VEGF2	Naked plasmid, percutaneous	Chronic myocardial ischemia	Angina improved significantly	[257]
Phase 0/I	VEGF2	Plasmid, percutaneous and intramyocardial	Chronic myocardial ischemia	Decrease episodes of angina, reduced ischemic area, improved perfusion	[452]
Phase I	FGF-4	Adenoviral, intracoronary	Angina pectoris	Exercise time and angina increased but not significantly	[259]
Phase III	FGF-1	Plasmid, intramuscular	Critical limb ischemia	No significant difference in limb survival	[265]
Phase I	FGF-1	Plasmid, intramyocardial	Critical limb ischemia	Decreased pain, improved ulcer healing, increased transcutaneous oxygen pressure and ankle-brachial index	[453]
Phase I	FGF-1	Plasmid, intramuscular	Critical limb ischemia	Improved ulcer healing and limb survival	[264]
Phase I/II	HGF	Plasmid, intramuscular	Peripheral artery disease	Reduced ulcer size, increase ankle-brachial index, improved rest pain and walking time. No dose-dependent effect observed.	[266,454]
Phase I	HGF	Plasmid, intramuscular	Critical limb ischemia	Improvement of rest pain and ulcer size	[267]
Phase I	HGF	Plasmid, intramuscular	Critical limb ischemia	Improved ankle-brachial index and ulcer healing, decreased pain	[268]
Phase I	HGF	Plasmid, intramuscular	Critical limb ischemia	Decreased pain	[455]
Phase I	HGF	Naked DNA, intramuscular	Critical limb ischemia	Improved ulcer healing and pain, increased oxygenation of limb	[456]
Phase II	$VEGF_{121}$	Adenoviral, intramuscular	Peripheral artery disease	No significant improvements	[260]
Phase I	$VEGF_{165}$	Plasmid, intramuscular	Critical limb ischemia	Higher doses led to improved healing of ulcers and less rest pain	[261]
Phase II	VEGF	Plasmid and liposome, percutaneous	Critical limb ischemia	Significant improved vascularity in VEGF-treated groups. No significant difference in ulcers, amputations or pain.	[262]
Phase I	$VEGF_{165}$	Naked plasmid, intramuscular	Peripheral artery disease	Inconclusive regarding dosage. Improved ulcer healing, pain and collateral neo-vessels	[457]
Phase I	$VEGF_{165}$	Plasmid, intramuscular	Critical limb ischemia	Improved skin ulcers and haemodynamic improvement. Limb survival and pain did not change significantly	[263]
Phase I	$VEGF_{165}$	Naked plasmid, intramuscular	Critical limb ischemia	Increase walking time	[458]
Phase I	HIF-1α	Adenoviral, intramuscular	Critical limb ischemia	High amputation rate but high adverse effects on placebo too, vision disorders, though improved ulcer healing is described	[269]
Phase II	HIF-1α	Adenoviral, intramuscular	Peripheral arterial disease	No significant differences in claudication, ankle-brachial index or quality of life measurements	[459]

also been demonstrated that exposure of EC to safe levels of ultrasound irradiation causes a down-regulation and redistribution of the VEGFR-2, increased proliferation and enhanced migration and sprouting in 3D culture [272].

The effects of low-intensity pulsed ultrasound (LIPUS) was also investigated in a porcine model of chronic myocardial ischemia. LIPUS therapy normalized global and regional myocardial function *in vivo*, increased capillary density and regional myocardial blood flow of the chronically ischemic region without any adverse effects, and enhanced protein levels of VEGF, eNOS, and FGF-2 in the ischemic myocardium without affecting those in the non-ischemic myocardium [273].

Among non-invasive techniques to promote angiogenesis, low-level laser therapy (LLLT) have shown promising results in skin wound healing and ischemic animal models.

Illumination with both blue and red light emitting diodes (LEDs) light can enhance the wound healing process in a skin flap model in rats by improving angiogenesis. Blue laser irradiation improves local tissue perfusion in a controlled manner by stimulating NO release from NO–haemoglobin complexes [274]. It has been shown that NO stimulates angiogenesis via stimulation of VEGF expression.

Another study showed that the LLLT enhanced the skin wound-healing effect of human ADSCs by enhancing survival of the ADSCs and stimulating secretion of growth factors in the wound bed in mice. The light-treated group was evaluated to exhibit rapid wound closure, a higher histological score and increased numbers of hair follicles and sebaceous glands in wound bed compared with the non-treated ADSCs [275]. Similarly LLLT treatment of ADSCs spheroids enhanced the functional recovery of the ischemic hind limb area in mice with respect to the regeneration of muscle tissue [276].

Pulsed electromagnetic fields (PEMF) have shown to enhance acute hindlimb ischemia-related perfusion and angiogenesis, associated with up-regulating FGF-2 expression and activating the ERK1/2 pathway in diabetic rats [277]. PEMF therapy improved postnatal neovascularization using a murine model of hindlimb ischemia. PEMF therapy enhanced ischemia-mediated angiogenesis, through up-regulating VEGF expression and activating the PI3K-Akt-eNOS pathway [278]. PEMF therapy was examined also in a rat myocardial infarction model. It prevented cardiomyocytes against hypoxia-induced apoptosis and preserved cardiac systolic function. Moreover PEMF induced angiogenesis and vasculogenesis through activating VEGF-eNOS system and promoting EPCs mobilized to the ischemic myocardium [279].

Currently, an ongoing clinical trial aims to induce cardiac functional regeneration in patients with ischemic CAD by combining the ability of extracorporeal shock waves to increase growth factor secretion and induce homing of progenitor cell to the target tissue for intracoronary cell therapy (source: www.clinicaltrialsregister.eu).

2.5. General approaches for delivery of therapeutic molecules

In vitro and preclinically, various delivery methods have been tested for numerous therapeutic molecules. Clinically, the route of administration is variable (Tables 2 and 3): for GF delivery, intracoronary or intra-arterial routes are the most common, while for genes, intramuscular/intramyocardial are preferred [138,140,255,264]. A summary of the

various delivery approaches tested preclinically and clinically can be found on Table 5. GFs have a very short-half-life following direct injection and their retention at the desired location cannot be ensured [71]. To extend the half-life of GFs, such as VEGF and FGF-2, sulphated macromolecules have been employed to emulate the physiological electrostatic interactions between growth factors and heparin and heparan sulphate proteoglycans [100,280], and sulphated polysaccharides like fucoidan. The sulphated moieties protect the growth factors from thermal and enzymatic degradation and limit their diffusion, therefore prolonging bioactivity. For instance, VEGF in scaffolds functionalised with heparin promoted blood vessel formation in mouse subcutaneous models [100,281]. Further, this non-covalent binding potentiates FGF-2 action as evidenced *in vitro* by increased HUVEC proliferation, migration and MMP-2 activation in a p38 and JNK dependent-manner, pathways that are strongly associated with tube formation [282].

Further to sulphated molecules, numerous materials have been utilised to fabricate scaffolds or functionalise them to further maintain the bioactivity of the therapeutic molecules, promote sustained release and ensure local retention. For growth factor delivery, for instance, FGF-2 has been delivered using gelatine microspheres to ischemic hindlimbs in canine models leading to improved capillary density [94]. PLGA nanoparticles loaded with VEGF have shown prolonged bioactivity in an aortic ring assay and increased number of small diameter (<400 μm) vessels in a mouse ischemic limb model [104]. In a similar model, VEGF has been delivered with alginate hydrogels and improved vascular density and prevented tissue necrosis [102].

Likewise, various materials have also been utilised as carriers for delivery of genes. For instance, PLGA nanospheres and collagen scaffolds have delivered VEGF plasmid DNA successfully to the skeletal muscle of ischemic limbs of mice [283] and promoted neo-vessel formation in the surrounding area of the construct in a subcutaneous model [284], respectively. Similarly, viral vectors have also been applied in combination with materials such as macro-porous PEG hydrogels, which facilitated cell infiltration *in vivo* and consequently improved vascularisation in mice [285].

Notably, as promising as the use of scaffolds might be preclinically, their clinical use represents a significant regulatory hurdle and further studies are required to assess efficacy and safety. Further, very few growth factors have been assessed in clinical trials and for the ones that have been evaluated (e.g. FGF-2, VEGF, PDGF), the results have not always been conclusive. Such results can derive from several issues such as the lack of testing of sustained release systems and the desensitisation of chronic ischemic tissue to growth factor treatment [286].

Given the limitations of protein delivery, genetic transfer is a suitable alternative. It facilitates localised overexpression of a relevant molecule for a prolonged period, thereby circumventing issues with protein half-life and local retention. For these reasons, there has been a higher number of human trials assessing transfer of several genes (e.g. VEGF, FGF-1, FGF-4, HGF, HIF-1α) for different conditions (e.g. myocardial and limb ischemia). Nonetheless, there are concerns with inducing unregulated overexpression of some molecules (e.g. VEGF). The delivery of plasmid DNA has been so far promising, given its low immunogenicity and low oncogenic risk, yet, further studies are necessary to assess long term safety and ensure transgene expression for a sufficient therapeutic period.

3. Cells as drugs

The use of single molecules or combinations thereof has produced promising outcomes *in vitro*, preclinically, and to a limited extent, clinically. However, in view of the various cell types involved in angiogenesis and their continuous cross-talk involving a complex secretome, this approach appears quite constrained. Theoretically, adding a relevant cell source to an ischemic tissue site, would locally introduce a modulating secretome, influencing cellular responses in the immediate vicinity [287–289]. This therapeutic mode can be tested by delivering conditioned media, cells (preconditioned or not), cell-derived vesicles (e.g. exosomes) or cells overexpressing a molecule of interest to the site of interest [290–292]. Relevant cells for angiogenic strategies not only comprise EC and EPCs but also BMSCs, which have been hypothesised to function as pericytes, ADSCs and BM-MNCs [53,54,293,294]. An important aspect in this regard is the presence of external microenvironmental factors that steer cell behaviour and, consequently, their secretome. Therefore, different modulators, such as hypoxia, mechanical stimulation and electromagnetic field stimulation can be used to pre-condition cells for subsequent *in vivo* and clinical use. The same challenge as with pharmacological agents arise, namely that of generating locally efficacious levels of active substances. Therefore, local retention of cells and their secretome need to be ensured, either by preventing the emigration of cells from the site of action into surrounding tissue, or their retrograde loss via puncture canals. To overcome this, materials such as collagen and fibrin, have been utilised to enable not only confinement of cells but also their survival by providing initial attachment support structures [295–297]. In this section, we will analyse *in vitro*, preclinical and clinical studies that have investigated the delivery of untreated and pre-conditioned cells, and cell secretome delivery.

3.1. Delivery of naïve cells

Cell delivery is an attractive approach for pro-angiogenic therapies envisioning a range of paracrine factors that are secreted locally in response to host tissue signals. Similar to locally administered pharmacological agents, cells have been delivered topically in animal models of hindlimb ischemia, myocardial infarction, stroke, and skin wound healing. The majority of cell based therapeutic approaches for angiogenesis tested clinically have been focused on peripheral arterial disease, a few studies have investigated coronary artery disease and myocardial infarction (Table 4). The preferred cell types investigated in these models include progenitor cells, EPCs, BM-MNCs, MSCs or ADSCs [298].

3.1.1. Endothelial progenitor cells (EPCs)

Since EPCs directly contribute to revascularisation by differentiating into ECs, they are a promising source, although their extraction procedure is more laborious than other stem cell sources. They can be harvested from human umbilical cord blood, adult bone marrow, peripheral blood and human foetal liver [299,300]. The mechanism of action of EPCs in promoting angiogenesis occurs by homing and direct incorporation into the existing vasculature, facilitating the growth of new capillaries, and by paracrine effects, through the secretion of cytokines and other proangiogenic growth factors, thus stimulating resident ECs to proliferate within the vascular wall [301,302]. EPCs express CD133, CD34 and VEGF receptor-2 (VEGFR-2), also called Flk-1 [303,304]. They also produce survival factors for ECs such as platelet endothelial cell adhesion molecule-1 (PECAM-1 or CD31), VE-cadherin, von Willebrand factor and eNOS and encourage endogenous EC to migrate and proliferate [305–307]. Upon entry into the circulation, EPCs differentiate into mature EC expressing CD144 (VE-cadherin) and von Willebrand factor, while losing CD133 and subsequently CD34 [305,308].

Cultured EPCs demonstrated positive results when transplanted into murine hindlimb ischemia models. Blood flow recovery and capillary density were noticeably improved and the rate of limb loss was significantly reduced after transplantation of human peripheral blood–derived EPCs [309–311]. These cells have also been transplanted in a bioactive nanofibrous scaffold, composed of collagen, polycaprolactone and bioactive glass nanoparticles, to full-thickness wound model in rats and significantly enhanced the formation of blood vessels by upregulating expression of HIF-1α, VEGF, and stromal cell-derived factor 1 alpha (SDF-1α) [312].

Some studies have translated the therapeutic potential of EPCs seen in animal models into human clinical trials using population of EPCs

Table 4
Clinical studies describing cell delivery.

Phase	Cells	Delivery method	Disease	Outcome	Reference
Pilot	Autologous bone marrow (BM) cells	Local implantation into the ungraftable area	Severe ischemic heart disease	Improved coronary perfusion	[320]
Phase I/IIa	$CD34^+$ cells	Intramuscular injection	Buerger's disease, critical limb ischemia	Reduced pain and ulcer sizes, improved $TcPO_2$ and pain free walking distance	[313]
Pilot	$CD34^+$ cells	Intramuscular injection	Critical limb ischemia	Reduced amputation rate, improved walking time	[314]
Phase I	Peripheral blood $CD133^+$ cells	Intramuscular injection	Critical limb ischemia	Reduced amputation rate, and rest pain, trend for improvement in pain-free walking time	[315]
Phase I/IIa	BM-MNCs	Intra-arterial injection	Critical limb ischemia	Improved neovascularisation	[28]
Pilot, Phase I	BM-MNCs vs Peripheral blood-MNCs (PB-MNCs)	Intramuscular injection	Unilateral ischaemia of the leg	Improved ABI, $TcPO_2$, rest pain, pain-free walking with BM MNC vs. PB MNCs	[317]
Pilot	BM-MSCs vs BM-MNCs	Intramuscular injection	Diabetic critical limb ischaemia	Improved ulcer healing, ABI and TcO_2 in BM MSC	[461]
Phase II	BM-MNCs	Intramuscular injection	Chronic limb ischaemia	Improved rest pain and ulcer healing	[462]
Phase I/II	BM-MNCs	Intramuscular injection	Critical limb ischaemia	Reduced amputation rate	[463]
Pilot	BM-MNCs	Intramuscular injection	Critical limb ischaemia	Non-significant trend for reduced amputation rate vs. placebo	[464]
Pilot, Phase I, Phase I/II	BM-MNCs	Intramuscular/intra-arterial injection	Critical limb ischaemia	Improved rest pain, ulcer healing, ABI with BM MNC	[465–467]
Phase II	BM-MNCs	Intra-arterial injection	Critical limb ischemia	No changes in ankle-brachial pressure. Improved ulcer healing and reduced pain after repeat injections	[326]
Phase I	BM-MNCs	Intramuscular vs intra-arterial injections	Critical limb ischemia	Higher CD34+ cell count related with limb salvage. No control group was utilised and no conclusion regarding the injection methods.	[468]
Phase I	BM-MNCs	Intramuscular injection	Critical limb ischemia	Comparable improvements in rest pain and in TcO_2 with cell therapy and control groups	[469]
Phase I/II	BM-MNCs	Intramyocardial and intracoronary injection	Acute myocardial infarction	Changes in the LVEF were significantly higher than at baseline in both groups, but not significantly difference between groups	[322]
Phase II	BM-MNCs	Intramyocardial injection	Ischemic heart failure	Cell therapy failed to improve LVEF, but reduced myocardial scar size	[321]
Phase I	BM-MNCs + VEGF	Intramuscular injection	Critical limb ischaemia	Cell therapy group exhibited increased ABI and ulcer healing	[470]
Pilot, Phase I	PB-MNCs	Intramuscular injection	Critical limb ischaemia	Improved rest pain, ulcer healing, blood perfusion, ankle-brachial pressure index	[471,472]
Phase I/II	G-CSF-mobilized PB-MNCs vs BM-MNCs	Intramuscular injection	Lower limb arteriosclerosis obliterans	Improved rest pain and ABI with G-CSF-mobilized PB-MNCs vs. BM	[323]
Phase II	BMSCs	Intramuscular injection	Critical limb ischaemia	Reduced rest pain and improved ulcer healing with high dose BM-MSCs	[333]
Phase I/II	BMSCs	Intracoronary injection	Acute myocardial infarction	Improved left ventricular ejection fraction (LVEF), infarct size and remodelling with BM-SCs transplantation compared to standard therapy	[334–336]
Phase I/II	BMSCs	Intravenous infusion	Ischemic stroke	Improved Rankin score and Barthel index	[338]
Phase I	BMSCs	Intravenous infusion	Ischemic stroke	No changes in functionality scores or imaging	[339]
Phase II	BMSCs	Intracoronary injection	Myocardial infarction	BM-SCs did not augment recovery of global LV function, but could favourably affect infarct remodelling.	[473]
Phase I	BMSCs	Intramuscular injection	Chronic non-healing ulcers (diabetic foot and Buerger's disease)	Reduced ulcer size and improved pain-free walking time	[474]
Phase I/II	BMSCs	Trans-endocardial injection	Ischemic cardiomyopathy	Improved LVEF	[337]
Phase I	ADSCs	Trans-endocardial injections	Ischemic cardiomyopathy	No significant changes	[348]
Pilot	Peripheral blood $CD34^+$ cells	Intracoronary transfusion	End-stage diffuse coronary artery disease	Significantly and progressively increased neovascularization	[475]
Pilot	$CD34^+$	Intramuscular injection	Critical limb ischaemia	Decreased amputation rate	[314]
Phase I	Circulating blood PCs	Intra-arterial injection	Critical peripheral arterial occlusive disease	A significant increase in the ABI, TcO_2 was seen in the CPCs group	[476]
Phase I	Autologous skeletal myoblasts	Intramyocardial injection	Left ventricular dysfunction or myocardial infarction	Myoblast transfer did not improve regional or global LV function beyond that seen in control group	[477]
Phase II/III	Cardiopoietic stem cells	Endomyocardial injections	Chronic heart failure	LVEF was improved by cell therapy vs standard care alone	[478]

enriched for CD34+ or CD133+. Clinical trials have assessed the safety of autologous CD34+ cells in patients with limb ischemia. Intramuscular injections of CD34+ cells led to improved toe brachial pressure index, pain scores, walking distance and reduced amputation rates [313,314].The effect of autologous CD133+ cells via intramuscular injection was also evaluated in a phase I trial in patients with Buerger's disease (1 patient), atherosclerosis (7 patients) or thromboembolic disorder (1 patient) [315]. Cell injection prevented leg amputation in seven of the nine patients and improved pain-free treadmill walking time and exercise capacity at 12 months.

3.1.2. Mononuclear cells (MNCs)

Mononuclear cells constitute mixed populations that can be derived from bone marrow, peripheral blood or umbilical cord and can contain lymphocytes, monocytes, MSCs or EPCs, depending on their origin. They are particularly attractive for cell therapy because they can be extracted in large numbers and be used in autologous applications without further expansion [316]. These cells can give rise to functional EPCs as well as secrete a broad spectrum of angiogenic factors (VEGF, FGF-2

Table 5
Summary of approaches tested preclinically and clinically for the delivery of growth factors, genes and cells.

		Preclinical	Clinical
Growth factors	VEGF	Collagen scaffolds [479,480]	Injection [135]
		Alginate hydrogel [102]	
		PLGA nanoparticles [104]	
		Heparin/PEG hydrogels [100,280]	
		Gelatine/heparin hydrogel [481]	
		Poly(vinyl alcohol) fibre mats with PLGA nanoparticles [101]	
		PLGA/alginate scaffolds [103]	
	FGF-2	Gelatine microspheres [94]	Injection [139]
		Fibrin scaffolds [107]	Heparin/alginate capsules [143]
		Heparin/alginate gel [109]	
		Gelatine microspheres in collagen cellulose scaffolds [481]	
		PCL/heparin conjugated gelatine fibrous matrices [106]	
		Poly(N-isopropylacrylamide-*co*-propylacrylic acid-co-butyl acrylate) hydrogel [111]	
		Decellularised pericardial ECM hydrogel [482]	
	PlGF-1	Nanofibers with liposomes [114]	
	PDGF-BB	PLGA microspheres in PLLA nanofibers [483]	
	Multi-delivery	Fibrin gel with heparin coacervate [125]	
		PLG scaffold and microspheres [126]	
		Alginate hydrogels [131]	
		Cellulose hollow fibres [132]	
		Alginate sulphate/alginate scaffolds [133]	
		Dextran hydrogels [484]	
		poly(ether)urethane–polydimethylsiloxane [485]	
		Cellulose acetate hollow fibres [486]	
		Gelatine nanoparticles in collagen/hyaluronic acid fibres [487]	
Gene	VEGF	PLGA nanosphere [283]	Injection [451]
		Collagen scaffold [284]	Liposome [262]
		Fibrin gel [228]	
		Macroporous PEG hydrogel [116,285]	
		Porous hyaluronic acid hydrogel [488]	
	Multi-delivery	Gelatine hydrogel [489]	
		Albumin microspheres in fibrin hydrogel [490]	
Cells	Myoblasts	Fibrin glue [295]	Injection [477]
		Cell sheet [438]	
	Cardiomyocytes	Collagen scaffold [296]	
		Cell sheets [437]	
	EPCs	Collagen/PCL/bioglass nanoparticles nanofibrous scaffold [312]	
	MNCs	Hyaluronic acid hydrogel [319]	Injection [321]
		Decellularised myocardium in fibrin gel [491]	
	BMSCs	Collagen/fibrin/dextran sulphate microcapsules [294]	Injection [334]
		Pullulan/collagen hydrogel [492]	
		Cell sheet [439]	
	ADSCs	Fibrin glue [346]	Injection [348]
		Decellularised small intestine submucosa, acellular dermal matrix, collagen/chondroitin sulphate/hyaluronic acid scaffold [493]	
		Methacrylated glycol chitosan hydrogel [494,495]	
		Cell spheroids [496]	

and Ang-1) or angiogenic cytokines (interleukin 1β and tumour necrosis factor α) [317].

The use of autologous bone marrow MNCs containing EPCs augmented neovascularisation and collateral vessel formation in a rabbit ischemic limb model [302]. After direct transplantation of bone marrow MNCs into ischemic heart muscle after coronary ligation in mini pigs, a local enhancement of angiogenesis was achieved [318]. Further, umbilical cord - MNCs have been evaluated in combination with a hyaluronic acid hydrogel in a porcine myocardial infarction model and showed significantly decreased scar area and promoted vessel formation in the infarcted region [319].

In clinical trials, injection of autologous bone marrow MNCs to ungraftable areas of the myocardium (no graftable coronary arteries were available, although the target myocardium remained viable) led to increased coronary perfusion in 3 out of 5 patients [320]. Intracoronary injections of MNCs have also reduced myocardial scar size but failed to improve LVEF [321]. However, combined intracoronary and intravenous MNCs injections led to improved LVEF in myocardial infarction patients [322]. In clinical trials for treatment of critical limb ischemia, intramuscular injections of peripheral blood MNCs showed improved ankle-brachial index (ratio between blood pressure at the ankle and at the upper arm; lower ratios suggest blockages due to peripheral artery diseases) and reduced rest pain when compared with bone marrow MNCs, though no significant changes were found in terms of ulcer healing rate and amputation rate [323]. Successful results obtained with peripheral blood MNCs have been associated with the secretion of angiogenic cytokines VEGF and HGF [324,325]. Other studies describing intra-arterial injections for treatment of critical limb ischemia present contradictory results. BM-MNCs have shown improved neovascularisation [28] but have also been unable to improve ankle-brachial pressure, although after repeated injections, improved ulcer healing and reduced pain was observed [326].

3.1.3. Bone marrow (BMSCs) and adipose derived stem cells (ADSCs)

BMSCs and ADSCs also constitute relevant sources for angiogenic cell therapies due to their ease of extraction, accessibility in large numbers and endothelial differentiation potential. The therapeutic effect of BMSCs in angiogenesis includes the paracrine effects associated with the secretion of various cytokines like VEGF, IL-6, platelet-derived growth factor, FGF, HGF and SDF-1, all of which stimulate ECs to migrate, differentiate and proliferate [327–329]. Direct injections of BMSCs into infarcted myocardium and ischemic thigh muscle have

Table 6
Summary of different cell pre-conditioning approaches.

Type of pre-conditioning		Conditions	Cell types	Main outcomes
Biochemical	Hypoxia	0%	Peripheral blood MNCs [56]	Increased secretion of proangiogenic factors (e.g. HIF-1α, VEGF, CD31, VEGFR2, Ang1)
		0.5%	BMSCs [375,376,497,498]	
		1%	Umbilical cord MNCs [373]	Improved blood flow in ischemic hindlimb, myocardial infarction and ischemic stroke animal models
			Umbilical cord MSCs [374]	
		2%	Peripheral blood MNCs [372]	
			BMSCs [375,377]	
Physical	Mechanical	Uniaxial stretch	ECs [382]	Inhibition of cell apoptosis
			HUVECs [57,499,500]	Increased tube formation
			Dermal fibroblasts [385]	Increased expression of pro-angiogenic factors (e.g. VEGF, CTGF, MMP-1, MMP-2)
				Preferential cell alignment
		Multiaxial stretch	Muscle derived stem cells [384]	Improved cardiac contractility and decreased fibrosis in myocardial infarction animal models
			HUVECs [501]	
			Coronary artery ECs [502]	
			Bladder smooth muscle cells [503]	
		Compression	BMSCs [383]	
		Stretch and fluid shear flow	Aortic ECs [387,388]	
	Electromagnetic	Electromagnetic field stimulation (EMF)	ECs [389]	Increased cell proliferation and tube formation in vitro
			HUVECs [504]	Inhibited hypoxia-induced apoptosis
		Pulsed electromagnetic field stimulation (PEMF)	HUVECs [66,279,390]	
		Extremely low EMFs	HUVECs [391]	

shown improved blood vessel formation in rat myocardial infarction and hindlimb ischemia models [330,331]. In the latter study, BMSCs isolated via plastic adherence and expanded until passages 4–5, were compared to BM-MNCs, extracted with a Ficoll density gradient centrifugation. Both BMSCs and BM-MNCs induced angiogenesis, but BMSCs induced higher capillary density and stronger perfusion than BM-MNCs. The transplanted BMSCs frequently differentiated into EC compared with transplanted BM-MNCs (based on the percentage of PKH26/von Willebrand factor -double-positive cells), and only BMSCs differentiated into vascular cells in ischemic muscle tissue. Additionally, *in vitro* studies showed that BMSCs secreted larger amounts of angiogenic factors compared with the amounts secreted by BM-MNCs and, when incubated under serum-free and hypoxic conditions, the majority of cultured BM-MNCs revealed cytoplasmic shrinkage, disintegration into small vesicles, and membrane blebbing. In contrast, these morphological changes were rarely observed in BMSCs. Furthermore, BMSCs have also been shown to promote vascularisation when injected intradermally in a mouse splinted skin wound model [332]. BMSCs were not found in the vascular structures, but in close proximity. This led the researchers to evaluate the paracrine effect of these cells in angiogenesis and found that BMSCs-conditioned medium promoted endothelial tube formation and that BMSCs expressed high levels of VEGF and Ang-1 but not Ang-2, suggesting that BMSCs engrafted in the wound released pro-angiogenic factors, which were partially responsible for BMSC-mediated enhanced angiogenesis.

The promising data seen with BMSCs *in vitro* and *in vivo* has prompted clinical studies. BMSCs have been shown to reduce rest pain and improve ulcer healing after intramuscular injection in patients with critical limb ischemia [333]. Myocardial infarction patients that received BMSCs through intracoronary injections have shown reduced infarct size and improved left ventricular ejection fraction [334–336]. Similarly, trans-endocardial injections have led to improved left ventricular ejection fraction in ischemic cardiomyopathy patients [337].

BMSCs have also been assessed as therapeutic modality in ischemic stroke. Delivery of BMSCs through intravenous infusion has shown variable outcomes. One study describes infusion of 10^8 cells (half in the beginning of the study and the other half 2–4 weeks later), using peripheral catheters, in patients that had presented symptoms in the last 7 days [338]. Patients treated with BMSCs ($n = 5$) showed a trend for improvement on the modified Rankin score and Barthel index, a scale that measures the degree of independence of stroke victims when performing daily activities. Another report of intravenous infusion of BMSCs, used a single bolus of $50–60 \times 10^6$ cells, on patients with stroke symptoms detected in the last 3–12 months [339]. Patients treated with BMSCs ($n = 6$), showed no changes in clinical scores of functionality or brain imaging. Given that both studies used the same delivery route, it is likely that differences in outcomes are related with cell dosing and time of intervention, given that improvements were seen when treatment was applied earlier with larger amounts of cells. Nonetheless, both studies had small sample sizes and ischemic stroke patients are quite heterogenous. It should be pointed out, that cells delivered intravenously typically end up in the lungs [349]. Therefore, direct cellular effects in the ischemic brain areas are unlikely to occur.

ADSCs secrete multiple angiogenic growth factors and cytokines such as VEGF and HGF that activate migration and proliferation of EC for new vessel formation [340,341]. Research shows that ADSCs use their paracrine potential for induction of angiogenesis and support collateral vessel remodelling [342]. ADSCs have been found to improve cardiac function, attenuate anterior wall thinning, reduce fibrosis and increase peri-infarct density of both arterioles and nerve sprouts in a rat myocardial infarction model [343]. In hindlimb ischemic mouse models, ADSCs injected intravenously restored blood flow to the affected limb [123,344,345]. The implantation of ADSCs induced angiogenesis not through endothelial differentiation of these cells, but by chemokines such as SDF-1. Additionally, ADSCs in a fibrin scaffold improved arteriole density and heart function in infarcted rat hearts [346].

In a clinical phase I/II study, intracoronary infusion of ADSCs was performed in patients with acute anterior wall myocardial infarction [347]. At 6 months follow up, cardiac magnetic resonance imaging showed a trend towards an improved left ventricular ejection fraction as compared to the placebo group, accompanied by a significant reduction in the perfusion defect and infarct size. Another clinical trial regarding transendocardial injection of ADSCs in patients with refractory ischemic cardiomyopathy was also performed. Injection of ADSCs preserved left ventricular mass and the regional wall motion and improved myocardial perfusion [348]. Moreover, a phase I study used intramuscular injection of autologous ADSCs to treat patients with critical limb ischemia. The ADSC injection resulted in improvement of transcutaneous oxygen pressure ($TcPO_2$), pain free walking time and ulcer size [349].

Cell therapy for lymphangiogenesis has been gaining interest and, although there are results available on *in vivo* and clinical studies, further work is needed to determine optimal cell source and lymphatic phenotype control [350]. Although, microvascular EC and lymphatics EC have shown to induce reduction of lymphoedema and increase in lymphatic vessel density in a rat tail model [351], stem cells appear to be the favoured source for most studies. ADSCs have shown increased VEGF-C expression and enhanced recruitment of M2 macrophages in a rat tail model [352] and increase of vessel density and expression of LYVE-1, VEGF-C and VEGFR-3 in a mouse hind limb oedema model [353]. Pre-conditioning of ADSCs *in vitro* with VEGF-C prior to implantation increased expression of VEGF-A, VEGF-C and Prox-1 which led to improved lymphangiogenesis in mice. Further enhancement of lymphangiogenesis was observed after TGF-β1 signalling inhibition, given its association with decreased proliferation and migration of lymphatic EC [354]. Culture of BMSCs with lymphatic EC supernatant led to enhanced expression of VEGFR-2, VEGFR-3 and podoplanin and decreased oedema in a rat model [355]. Cells have also been delivered together with growth factors. ADSCs and BMSCs have been combined with VEGF-C and showed improved lymphatic vessel density in mouse dermal oedema and rabbit hindlimb oedema, respectively [356].

Given the promising results seen in preclinical tests, clinical testing of delivered cells has also been performed in women with secondary lymphoedema derived from breast cancer surgery and radiotherapy. Intramuscular injection of ADSCs was conducive to a similar reduction of limb swelling when compared with compression sleeve therapy (CST). However, CST led to reoccurring swelling, while ADSCs injection promoted a more stable outcome [357]. Conversely, delivery of BMSCs reduced pain and lymphoedema volume significantly when compared with decongestive physiotherapy [358].

3.1.4. Pericytes and blood-derived angiogenic cells

Pericytes are specialised cells that are reside wrapped around capillaries and are crucial for vessel maturation and proper function [359,360]. Although there are no fully pericyte-specific markers, a PDGFR-ß and/or α-SMA, desmin, PDGFR-β, aminopeptidase N and high molecular weight melanoma antigen (known as NG2 in mice) have been proposed as pericyte signature in combination with their perivascular location [361,362].

The origin of pericytes is controversial [361]. Some research groups have proposed that pericytes are a differentiated subpopulation of MSCs [293,363], others have found monocyte-derived cells to express pericyte-markers and to attain a typical location around blood vessels [294,364].

The intramyocardial transplantation of mesenchymal pericytes, derived from skeletal muscle, into a myocardial infarct has shown reduced fibrosis and improved cardiac function in mice [365]. Simultaneously, pericytes, purified from the saphenous vein, supported cardiac function and modulated cardiac wall thinning, while also enhancing angiogenesis in the peri-infarct and infarct area by reducing endothelial cell apoptosis and supporting endothelial cell proliferation [366]. Blood-derived pericyte like cells have been described; they can be derived from MNCs derived from buffy coats [294]. With a specific culture protocol (exposure of buffy coats to fibronectin coating and application of pulsed macromolecular crowding), blood-derived angiogenic cells (BDACs) with pericyte characteristics have been produced that in Matrigel assays tend to cluster at branch points of HUVEC tubular networks and migrate along endothelial tubes in this system. Moreover, the injection of BDACs in the calf muscle of mice with hind limb ischemia reduced the inflammation, fibrosis and adipose replacement in the affected muscle tissue.

3.2. Delivery of pre-conditioned cells

Naïve cells, (i.e. cells that have not been exposed to any form of pre-conditioning or external stimuli during *in vitro* culture and expansion) are an appealing approach due to simplicity of harvesting and absence of cell culture. Therefore, they come with a lower regulatory threshold, but they can present issues related with low cell survival, inadequate secretion profile and differentiation towards other lineages after administration, which are not relevant to pro-angiogenic applications, therefore compromising functional recovery [367,368]. Therefore, microenvironmental pre-conditioning *in vitro* can help with overcoming some of these issues as means to modulate cell phenotype and secretion pre-implantation (Table 6). However, we need to keep in mind that this requires culture time and exposure of cells to factors and stimuli, which raises the regulatory bar.

3.2.1. Biochemical pre-conditioning

One of the most common stimuli assessed is hypoxia, given its prominent role in initiating angiogenesis. Adult tissues experience oxygen tensions that are considerably different from that in standard cell culture, where atmospheric oxygen tensions of 21% prevail. In human tissues, oxygen concentrations between 2%–9% are considered to constitute physiologic normoxia [369]. It is obvious, that conventional *in vitro* conditions expose the cells to a non-physiological hyperoxic environment, which creates stress. Indeed, lowering oxygen tensions in cell culture has proven to be beneficial, for example, in maintaining undifferentiated states of embryonic, hematopoietic, mesenchymal, and neural stem cell phenotypes and also influencing proliferation and cell-fate commitment [44]. Therefore, hypoxic preconditioning of stem cells *in vitro* can increase their motility and therapeutic potential prior to transplantation [370].

Hypoxia is known to stimulate VEGF expression in EC culture [371] but pro-angiogenic effects have also been reported on other cell sources.

Hypoxic pre-conditioning (anoxia) of peripheral blood MNCs improved their differentiation towards EPCs, as shown by increased expression of CD31, VEGFR-2 and VEGF. Further supplementation with VEGF under hypoxia also promoted migration of newly differentiated EPCs and improved blood flow in an ischemic hindlimb rat model [56]. Similar results were seen in a rabbit ischemic hindlimb model when peripheral blood MNCs were pre-cultured at 2% oxygen tension [372]. Umbilical cord MNCs pre-conditioning with 1% oxygen tension also promoted increased gene expression of Ang-1, COX-1, PIGF, and MCP-1 and reduced the expression of proinflammatory genes such as IL-1 and IL-20 in a mouse hindlimb ischemic model [373]. 1% oxygen tension also increased VEGF secretion in umbilical cord MSCs [374].

Further, pre-conditioning with various hypoxic conditions can promote the pro-angiogenic capability of BMSCs. Culture with BMSCs at 0.5% oxygen tension increased the expression of HIF-1α, VEGF, VEGFR2 and Ang1 leading to increased cell survival after implantation in a rat myocardial infarction model [375] and improved functional recovery in rats after ischemic stroke [376]. Further, BMSCs pre-conditioned with 2% oxygen tension, promoted neo-capillary formation in a rat cornea pocket model [377] and in a osteonecrotic rabbits [378], increased endothelial differentiation and reduced infarct area in rats [375].

Hypoxia preconditioning has shown promising results in EC, EPCs UC-MSCs and BMSCs, however, published oxygen tensions vary considerably, across cell types and even within one cell type. This highlights the need for standardisation and validation of protocol in order to create release criteria for cells to be used therapeutically.

3.2.2. Physical pre-conditioning

3.2.2.1. Mechanical stimulation. In blood vessels, EC are exposed to various mechanical stimuli internally and externally which regulate angiogenesis [379,380]. Mechanical forces regulate capillary morphogenesis and modulate EC phenotype, cytoskeletal reorganisation and vascular remodelling through shear stress from blood flow cyclic strain and from circumferential vessel deformation [381,382]. Cyclic strain also inhibits EC apoptosis, increases EC proliferation. It also enhances expression of MMPs and membrane-type MMPs suggesting a role in

neovessel formation. The use of cyclic stretch in HUVEC seeded in a collagen gel significantly increased formation of invasive structures *in vitro* [57]. Combining stretching with growth factor supplementation, demonstrated an additive effect when higher strains were combined with FGF-2 or VEGF.

Furthermore, mechanical stimulation of BMSCs enhanced tube formation in a three-dimensional matrix by upregulating the pro-angiogenic MMP-2, which is involved in early angiogenic events associated with ECM remodelling and EC migration, particularly through the exposure of a cryptic epitope of collagen IV or cleavage of growth factors, such as pro-TGF-β or VEGF. [383].

Transplantation of mechanically stimulated muscle-derived stem cells (MDSCs) into mice with acute myocardial infarction showed improved cardiac contractility, increased numbers of host $CD31^+$ cells, and decreased fibrosis, in the peri-infarct region, compared to hearts treated with non-stimulated MDSCs [384]. Mechanical strain prevented apoptosis and increased the adhesive ability of dermal fibroblasts *in vitro*, conferring a survival advantage *in vivo* after transplantation into full-thickness wounds of diabetic mice. Cyclic stretch stimulated cellular survival mediated by the activation of extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs) and the Akt. The stretched fibroblasts also showed increased expression of VEGF and stromal cell-derived factor-1α (SDF-1α) [385], which would support local angiogenesis.

Cells pre-conditioned with mechanical stimuli are yet to be assessed in depth in preclinical and clinical settings. However, at the *in vitro* stage, there are increasingly complex bioreactors under development combining stretch and fluid shear flow [386–388], for instance, which will contribute to improve the understanding of the role of mechanical forces on angiogenesis.

3.2.2.2. Electromagnetic stimulation. Electromagnetic field stimulation (EMFs) and pulsed electromagnetic field (PEMFs) pre-conditioning promote angiogenesis. EMF facilitates EC proliferation and formation of sprout-like structures *in vitro* in a 2D scratch assay [389]. PEMF increases EC proliferation and tube formation *in vitro* in fibrin gels. Moreover, the addition of conditioned media from PEMF-treated HUVEC to fibroblasts or HUVEC cultures increased proliferation and migration, even though when applied directly to fibroblasts, PEMF had not induced any changes. The authors proposed that the pro-angiogenic effects were related with increased FGF-2 secretion [390]. Another study demonstrated that extremely low frequency (ELF) EMFs increased HUVEC proliferation and tube formation in Matrigel system by acting on VEGF transduction pathways [391]. Similar reports have shown that PEMF inhibited the process of hypoxia-induced apoptosis and augmented tube formation, migration and proliferative capacities of HUVEC acting on the Akt-eNOS-VEGF pathway [66,279].

It has been proposed that PEMF act by mediating activity of voltage gated calcium channels, through stimulation of NO synthesis, facilitated by Ca^{2+} and calmodulin or through transient receptor potential channels (TRPs), similarly to other forms of mechanical stimulation [393,394]. This explains some of the working mechanisms of PEMF and provides a rationale for exploring this technology further.

3.3. Delivery of genetically modified cells

Certain factors such as donor age, comorbidities and effects of *ex vivo* culture can affect cell potency, phenotype and consequently, secretion profile [395]. However, the efficacy can be improved by manipulating cellular paracrine activity by genetic manipulation to increase the expression of a molecule of interest. Pre-implantation genetic transfer has been explored in both permanently differentiated cells and stem cells. The most common approaches are based on viral gene delivery (retroviral, adenoviral and lentiviral) and are currently under evaluation for several clinical indications. Given its key role in initiating angiogenesis, VEGF is still a preferred target. Myoblasts transduced with VEGF were injected in a porcine myocardial infarction and implanted in mice ischemic limb models where they were shown to promote increased vessel density and blood perfusion [396,397]. Keratinocytes overexpressing VEGF in a collagen/GAG material have also been utilised to accelerate wound healing through enhanced blood vessel formation, expression of CD31 and improved blood flow recovery [398]. Viral transfection of Id1 has been performed in HUVEC which were subsequently injected intramuscularly in a mouse ischemic hindlimb model. Id1 is a helix-loop-helix transcription factor that plays a key role in embryonic vascular development and is highly expressed in tumour vasculature. Its overexpression in HUVEC has led to improved limb salvage over unmodified HUVEC, through Ang1 regulation [399].

Although differentiated cells have been utilised, most of the efforts have focussed on delivery of modified stem or progenitor cells with particular emphasis on BMSCs. In BMSCs, VEGF transfer using biodegradable polymeric nanoparticles, improved capillary density and limb salvage in the mouse ischemic hindlimb [400]. Furthermore, adenoviral delivery of VEGF to BMSCs has shown increased capillary density, reduction of infarct size and presence of cardiomyocytic phenotype with implanted cells integrated in the vasculature following intramyocardial injection in rat infarction models [401–403]. Additional studies focussing on myocardial infarction models have utilised retroviral delivery of protein Akt1, plasmid delivery of sonic hedgehog (Shh) and lentiviral delivery of LIM-homeobox transcription factor islet 1 (ISL-1) BMSCs. Akt1 has been associated with promotion of cell survival by acting through the phosphatidylinositide 3′-OH kinase (PI3K)/c-Akt kinase cascade. Injected Akt1-modified BMSCs were shown to reduce inflammation, myocyte hypertrophy, reduction of infarct size and improved cardiac function, although neo-vessel formation and blood flow recovery were not assessed [404]. Shh transfection into BMSCs was also shown to improve myocardial infarction recovery through improved cell survival post-injection mediated by protein kinase C (PKC) signalling, since upregulation of netrin-1 and iNOS were also observed, but also through subsequent upregulation of VEGF and angiopoietins [405]. ISL-1 overexpression in BMSCs has also been shown to improve cardiac function, decrease apoptosis and inflammation after intramyocardial injection in a rat model [406]. ISL-1 has an important role during cardiac development, is a marker for cardiac progenitors and is believed to promote vascularisation.

ADSCs have been employed to a lesser extent. Overexpression of VEGF in ADSCs, following intramuscular delivery led to increased Ang1 secretion, improved neovascularisation and reperfusion in a hind limb ischemic model [407]. Transfection of Ang1 showed improved capillary density, infarction size reduction and enhanced ventricle function after intramyocardial injection in a rat infarction model [408]. It was proposed that Ang 1 protected cardiomyocytes from oxidative stress induced apoptosis, since, *in vitro*, Ang-1 overexpressing ADSCs were shown to promote proliferation and survival of cardiomyocytes after exposure to hydrogen peroxide.

EPCs have also been a target of interest. EPCs overexpressing IGF-1 have shown increased capillary formation and cardiomyocyte proliferation and survival contributing to improved cardiac functional recovery in a rat myocardial infarction model, confirming previous reports of antiapoptotic and proliferative activity of IGF-1 [409]. In ischemic hindlimb models, overexpression of VEGF and kallikrein in EPCs has also led to increased capillary density and blood flow [222,410]. Expression of kallikrein has been associated with promotion of angiogenesis in skeletal muscle [411].

While there is extensive preclinical analysis available for genetically modified cells and clinical data regarding gene delivery, very little has been translated to the bedside. Nevertheless, autologous EC and SMCs modified by transfer of angiogenic genes ($VEGF_{165}$, Ang-1, eNOS) are currently under evaluation in patients with peripheral arterial disease. Preliminary results suggest that the administration is feasible and safe and has no apparent drug-related adverse events (NCT00390767; www.clinicaltrials.gov). A phase II study is currently assessing safety

and efficacy of EPCs transfected with human endothelial nitric oxide synthase (eNOS) to restore functional tissue in regions that otherwise would form only scar (NCT00936819; www.clinicaltrials.gov). Another phase II trial is also evaluating EPCs transfected with eNOS. This study aims to enhance lung microvascular repair and regeneration in patients with severe symptomatic pulmonary arterial hypertension (NCT03001414; www.clinicaltrials.gov).

Genetic engineering can increase the potential of therapeutic cell delivery by facilitating overexpression of angiogenic genes or anti-apoptotic factors. However, safe and efficient gene delivery has been challenging since transduction raises safety concerns such as immunogenicity, toxicity or mutagenesis [412], whereas the transfer efficiency of non-viral methods is still low [413]. Although overexpression of pro-angiogenic factors is relevant, current efforts are directed towards single gene transfer, which is likely to present similar issues to single growth factor delivery, particularly if VEGF or PDGF are used, given their association with formation of aberrant vascular structures and tumours. There is also a need for improvement of delivery strategies that improve cell survival. Several approaches were focussing of transfer of anti-apoptotic or mitogenic factors. However, the preferred method of delivery of genetically modified cells is the injection, which is associated with poor local cell retention and survival and relies on cells attaching to the site of injury for *in situ* action. Scaffolds to facilitate cell retention are currently more frequently used for the delivery of naïve cells than for genetically modified cells.

3.4. Delivery of cell secretome and cell-derived vesicles

As a counterpart to the local transfer of intact living cells, the local delivery of their secretome – either from conditioned media or cell-derived extracellular vesicle – is currently explored as a scalable and standardisable alternative. Conditioned media allow to evaluate the effects of a whole range of secreted soluble molecules, while exosomes not only carry proteins, lipids and nucleic acids, but also channels and receptors as membrane derivatives. Their cargo is not extensively characterised [292], but their receptor pattern will depend on the cell type they are derived from. For instance, UC-MSCs conditioned media promote HUVEC migration, network and branch point formation in Matrigel [66,414]. Conditioned medium has also been used to improve proliferation of stem cell sources [415]. BMSCs pre-treated with medium from healthy cardiomyocytes exposed to oxidative stress and high glucose, showed enhanced survival, proliferation, angiogenic ability and improved function in diabetic hearts [416]. Furthermore, the angiogenic effects of ADSCs and their conditioned media have been evaluated in a rodent hindlimb ischemia model. Even though ADSCs conditioned medium was not as effective as injected ADSCs on restoring blood flow, it still led to neo-vessel formation in the ischemic hindlimbs. Analysis of these conditioned media revealed presence of TGF-β, VEGF, granulocyte colony stimulating factor (G-CSF), FGF-2 and HGF [340]. Cell secretomes can also be applied using injectable carriers to prolong the half -life of its components. ADSC secretome delivered with an injectable nanocomposite hydrogel showed increased capillary density, reduced scar area and improved cardiac function after myocardial infarction in a rat model [417].

As with complete cells, microenvironmental pre-conditioning is a useful tool to modulate the cell secretome. For instance, media from BMSCs cultured under hypoxia has been shown to improve HUVEC adhesion *in vitro*, whilst conditioned media from EPCs supported HUVEC proliferation independently of the oxygen tension used, indicating that BMSCs and EPCs have different roles during angiogenesis [418]. Additionally, conditioned media derived from EPCs culture under hypoxia (1.5% oxygen tension) has also improved blood flow and restored muscle function in a rat hindlimb ischemic model in a comparable manner to injection of EPCs [62]. Conditioned media from mechanically stimulated BMSCs enhanced tube formation of EC in Matrigel. The paracrine effect of BMSCs was mediated by an upregulation of VEGFR and FGFR signalling cascade [419].

Exosomes are endosomal membrane vesicles with diameters of ~40–150 nm. They originate in the late endosomal compartment from the inward budding of endosomal membranes, which generates intracellular multi-vesicular endosomes (MVEs). Pools of exosomes are packed in the MVEs and released into the extracellular space after the fusion of MVEs with the plasma membrane. Exosomes therefore are present in conditioned media, and various methods have been developed to isolate and concentrate them for subsequent applications. There is emerging evidence that exosomes play an important role in intercellular communication by serving as vehicles for transferring various cellular constituents between cells [420–423].

Exosomes have advantages over the use of naked growth factors or conditioned media, because they possess a lipid bilayer that protects the content from proteolytic degradation, greatly prolonging the half-life of the cargo. Several studies demonstrated that exosomes derived from EPCs enhanced migration, proliferation and tube formation of EC *in vitro*. Furthermore exosomes treatment increased the expression of angiogenesis-related genes such as FGF1, VEGFA,VEGFR-2, eNOS, IL-8 by activating Erk1/2 signalling [424,425]. It was demonstrated that miR- 214, which controls endothelial cell function and angiogenesis, plays a dominant role in exosome-mediated signalling between ECs. Similarly, exosomes secreted by human ADSCs carrying miR-125a have been taken up by EC and significantly promoted angiogenesis *in vitro* and *in vivo*. miR-125a can repress the expression of the angiogenic inhibitor delta-like 4 (DLL4) and modulate endothelial cell angiogenesis through promotion of formation of endothelial tips [426]. BMSC exosomes were shown to enhance tube formation in HUVEC *in vitro* and to activate Akt, ERK 1/2 and STAT3 pathways in dermal fibroblasts which led to increased expression of HGF, IL-6, IGF-1, NGF and SDF-1 [427]. Additionally, BMSCs-derived exosomes have been shown to increase capillary network formation, blood flow, improved limb salvage and reduced necrosis in mice and rat hindlimb ischemic models [428,429]. Similarly to BMSCs, UC-MSCs - derived exosomes have also promoted the proliferation, migration, and tube formation of EC in a dose-dependent manner [430]. Moreover, BMSCs-derived exosomes restored bioenergetics, reduced oxidative stress and activated pro-survival signalling, thus enhancing cardiac function and geometry after myocardial ischemia model [431]. When injected into mouse ischemic hindlimb tissue, $CD34^+$ mononuclear cells derived exosomes improved ischemic limb perfusion and capillary density. These exosomes increased miR-126-3p levels, which enhanced angiogenesis by modulating the expression of angiogenic genes such as VEGF, Ang1, Ang2, MMP-9 [432].

In terms of wound healing, UC-MSCs – derived exosomes have improved healing and new blood vessel formation in a rat skin burn model. The exosomes promoted β-catenin nuclear translocation and increased expression of proliferating cell nuclear antigen, cyclin D3, N-cadherin, β-catenin and the decreased expression of E-cadherin, all mediated by Wnt/β-catenin signalling pathway [430].

Although the potential of cell secretome and cell-derived vesicles has been established in preclinical studies, their clinical evaluation is still in its infancy. An ongoing phase I clinical trial aims to evaluate the effect of exosomes derived from autologous plasma on intractable cutaneous ulcers (NCT02565264; www.clinicaltrials.gov). Another phase I study is underway to assess the administration of BMSCs-derived exosome enriched with miR-124 on improvement of disability of patients with acute ischemic stroke. It was hypothesised that intravenous administration of BMSC-generated exosomes post-stroke will improve functional recovery and enhance neurite remodelling, neurogenesis and angiogenesis (NCT03384433; www.clinicaltrials.gov).

The use of conditioned medium and exosomes still requires further characterisation, because their cargo and resultant therapeutic effect will vary depending on the cell source, culture method and use of pre-conditioning. However, they might present advantages over delivery

of cells in terms of upscaling, content and production control. Furthermore, exosomes offer the possibility of targeted delivery, due to the presence of membrane receptors, and of additional encapsulation of other relevant molecules [433].

3.5. General approaches for cell retention and survival after implantation

Delivery of cells to the site of interest presents therapeutic advantages, however, there are still drawbacks that need to be overcome. Current main issues include poor local cell retention and cell death due to the lack of attachment or presence of adequate survival factors. To address these issues several methods have been developed. There are basically two options, systemic administration (intravascular), or direct tissue injection of cells either in suspension or embedded in a carrier material. In a porcine myocardial infarction model, intracoronary infusion and endocardial injection gave better local cell retention in the heart than intravenous administration up to 14 days post-delivery [434]. Nonetheless, around 20% of injected cells were still found in the lungs after intracoronary and intravenous infusions. This clearly shows the need for approaches other than infusion of cell suspensions. For instance, BMSCs embedded in agarose, collagen and fibrin microcapsules have been delivered to rat infarcted myocardium to further promote their local retention and survival [297]. Collagen scaffold and a temperature responsive hydrogel, also for BMSC delivery, improved healing, neovascularisation and MMP-9 expression in a rat full thickness wound model and in a mouse diabetic ulcer model [435,436].

Although scaffolds present a relevant alternative, other methods have focussed on utilising cell secreted ECM as a support structure for the implanted cells. This has the advantage of maintaining intact cell-cell and cell-matrix interactions. Cardiac cells have been grown *in vitro* in temperature responsive dishes to fabricate cell sheets, which, when placed directly on the infarcted area in a rat myocardial infarction model, led to improved cell survival, localisation and higher number of mature capillaries than cell injections [437]. Temperature sensitive gels with RGD adhesive peptides have also been investigated for myoblast sheet creation and were shown to lead to improved cell retention and survival and perfusion recovery after implantation in a hindlimb ischemic model [438]. Magnetic nanoparticles can also be used *in vitro* to force cells into an organised cell sheet that can be implanted. BMSC sheets fabricated that way showed increased VEGF secretion and further increased capillary density and blood perfusion to ischemic muscle [439]. It was also observed that the BMSC sheets increased skeletal muscle cell survival in the vicinity, however, this might have come from the intrinsic anti-oxidative peroxidase-like activity of the magnetic nanoparticles.

In an attempt to create assemblies that are more complex than sheets, cells have also been organised into spheroids. HUVEC spheroids implanted in a mouse ischemic hindlimb model increased cell survival over monolayer-grown cells and expression of VEGFR1 and VEGFR2 [440]. In an analogous model, umbilical cord MSC spheroids also improved limb survival, decreased necrosis and increased expression of von Willebrand factor and NG2, indicating vessel maturation [441]. The use of such methods also allows for implanting co-cultured cells and positive results in terms of limb survival and blood perfusion recovery were observed in limb ischemic models when HUVEC and umbilical cord MSC aggregates were applied [442]. It was proposed that the encouraging outcome was based on the differentiation of umbilical cord MSCs into SMCs and stabilising the newly formed HUVEC tubular network, however, further work is needed to confirm these assumptions in a fully human system.

Many delivery vehicles are currently under preclinical investigation for the delivery of cells and therapeutic molecules. The preferred method in the clinical setting for treatment of peripheral arterial diseases is the intramuscular injection, while for cardiac ischemic diseases it is more variable: intravascular, intramyocardial or trans-endocardial. Intramuscular injections are a better option in limb ischemia, given that, due to the compromised blood flow, cells injected intravascularly are unlikely to reach the diseased tissue. Further, cells delivered intravascularly are typically found in the lungs, though it has been described that higher cell dosages or repeated cell injections can be conducive to higher amounts of cells reaching the target tissue. The latter stems from the idea that cells interact with endothelial cells in the lungs through VCAM-1 and, when high cell numbers are used, there is receptor saturation that prevents further cells from being trapped [443]. Nonetheless, cell/particle size is also an important parameter [444].

Intramuscular injections are advantageous as they can allow for cell entrapment in the tissue. However, cell survival will depend on the specific tissue conditions. Ischemic tissue is deprived of nutrients and oxygen, has higher amounts of lactic acid, free radicals and reactive oxygen species which can compromise cell survival and phenotype.

There are still significant regulatory hurdles for the clinical translation of biomaterials and cell-derived materials. Nevertheless, these come with the promise of functional repair beyound the current alternatives and must be fairly assessed. It is also important to consider that in many cases translation is not justified, as the preclinical models utilised do not always provide reliable information regarding safety and efficacy in humans.

4. Conclusions and future perspectives

Therapeutic angiogenesis is based on an exciting body of literature furthering the understanding of relevant mechanisms. There have been discrepancies between *in vitro* angiogenesis assays and *in vivo* data which can be partially attributed to using endothelial cells solely *in vitro*, while they are in reality influenced by crosstalk with non-endothelial cells *in vivo*. Nevertheless, remarkable progress has been made in the last decades with drug and cell delivery. As a new option, the use of the cell secretome has been met with growing interest. Although growth factors are unquestionably the most researched option due demonstrated efficacy in preclinical setting, issues arise regarding their cost, activity and need of suitable carriers. Their instability necessitates supraphysiological local concentrations and that can lead to undesired side effects in clinical settings, as exemplified by the case of PDGF. The use of small molecules should overcome most of those issues. As a scientifically attractive approach gene delivery has attracted much attention due to the promise of *in situ* sustained delivery of a protein of interest. However, previous fatal outcomes with adenoviral transfer have led to a re-evaluation of clinical gene therapy approaches. While this has not prevented continued animal experimentation, clinical evidence for efficacious angiogenic gene delivery has to be gathered yet. Regarding cell delivery, different permanently differentiated, progenitor and stem cell sources, undifferentiated, pre-conditioned or genetically modified, have been assessed. Although clinical evidence appears promising, additional efforts are required to evaluate the advantages of cell manipulation prior to implantation in full. Similarly, for the use of exosomes, significantly more *in vitro*, preclinical and clinical work is required to justify the labour and cost intensive extraction process. Additionally, given the complexity of angiogenesis, it is likely that further advancements will only come about with multicomponent delivery. In this regard, the most promising drug calls still might be prolyl hydroxylase inhibitors. Besides being stable small molecules, they tackle an angiogenic master switch (HIF-1α) and thus bring a variety of angiogenic factors into play simultaneously. This is a situation akin to multidrug/multigene or secretome application. Of note, some members of this compound class are already approved for clinical use and can be re-purposed in an indication-discovery approach for angiogenesis. While some encouraging results support the use of drugs and cells, their wider application is currently constrained to use as last resort in otherwise

intractable ischemic conditions. This and the high variability of treatment conditions (e.g. experimental treatments delivered alongside surgical grafting procedures) severely hinder proof of clinical efficacy and statistical significance of the studies. A clear consensus on the efficacy of the three therapeutic approaches in microangiogenesis we have discussed has not been reached yet. What is, however, clearly emerging is an overarching need for the development of appropriate delivery vehicles to ensure local retention and continued biologic activity at the site of interest of angiogenic compounds.

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