CELLULAR STRATEGIES TO PROMOTE VASCULARISATION IN TISSUE ENGINEERING APPLICATIONS

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Abstract

Vascularisation is considered to be one of the greatest challenges in tissue engineering. Different strategies exist but cell-based approaches have emerged as a promising therapy to achieve successful vascularisation. The use of endothelial cells to engineer vascularised tissues has been extensively investigated. This field of research has evolved with the discovery of endothelial progenitor cells, a subpopulation with a high regenerative potential. However, the survival of endothelial cell populations alone seems to be impaired. To overcome this problem, co-culture systems, involving supporting cells, like mural cells, fibroblasts, or more tissue-specific cells have been developed. Endothelial cells benefit from the extracellular matrix components and growth factors produced by the supporting cells, which results in neovessel stabilisation and maturation. The use of endothelial progenitor cells in co-culture systems appears to be a promising strategy to promote vascularisation in approaches of increasing complexity. Herein, the authors provide an overview of the cellular strategies that can be used for increasing vascularisation in tissue engineering and regeneration.

Keywords: Angiogenesis, cellular therapies, co-culture models, endothelial progenitor cells, regeneration.

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The Relevance of Vascularisation in Tissue Engineering

The regeneration of a lost or damaged tissue function in the adult generally involves a recapitulation of developmental processes, which consequently implies the replication of biological concepts and instructions expressed during the embryonic development (Lee *et al.*, 2011). This is the main reason why regeneration of large defects in a human adult tissue does not occur naturally.

Tissues are composed of several cell types, resulting in a highly organised complex structure, integrated into the body, namely through the vascular and the nervous systems. Tissue engineering has been evolving as an interdisciplinary science, which intends to restore, maintain or improve tissue function (Langer and Vacanti, 1993). In general terms, tissue engineering aims for the creation of adequate tissue or even organ equivalents, although, up to now, only tissue-engineered products for skin and cartilage regeneration have been successfully used in the clinics (Phelps and García, 2010). One explanation for this fact is based on the recognition that the demands of skin and cartilage in terms of blood supply are relatively reduced (and virtually non-existent in cartilage), owing to the fact that these tissues are supplied through diffusion from distant blood vessels. However, the maximum diffusion distance of nutrients and oxygen out of blood vessels is of about 200 μm (Carmeliet and Jain, 2000) and, due to mass transfer limitations, organs with a more complex three-dimensional structure actually need an integrated vascular network to support cell survival. Overall, the inability to engineer blood vessels *in vitro* for subsequent transplantation has been referred to as the main reason associated with the limited clinical success of tissue engineering strategies (Lovett *et al.*, 2009; Phelps and García, 2010; Novosel *et al.*, 2011).

When a tissue engineered construct, including transplanted cells, faces an insufficient blood stream, associated problems arise that result in functional limitations of the bioengineered tissue or even lead to the failure of the implanted construct. Hence, several strategies have been investigated with the aim of promoting vascularisation in tissue engineering applications, namely growth factor delivery, cell transplantation, and the use of materials for immobilisation strategies (scaffold-based approaches), which have been extensively reviewed (Rouwkema *et al.*, 2008; Lovett *et al*., 2009; Phelps and García, 2010; Novosel *et al*., 2011). In fact, there have been different efforts to develop new therapies so that a faster and successful vascularisation is achieved. This

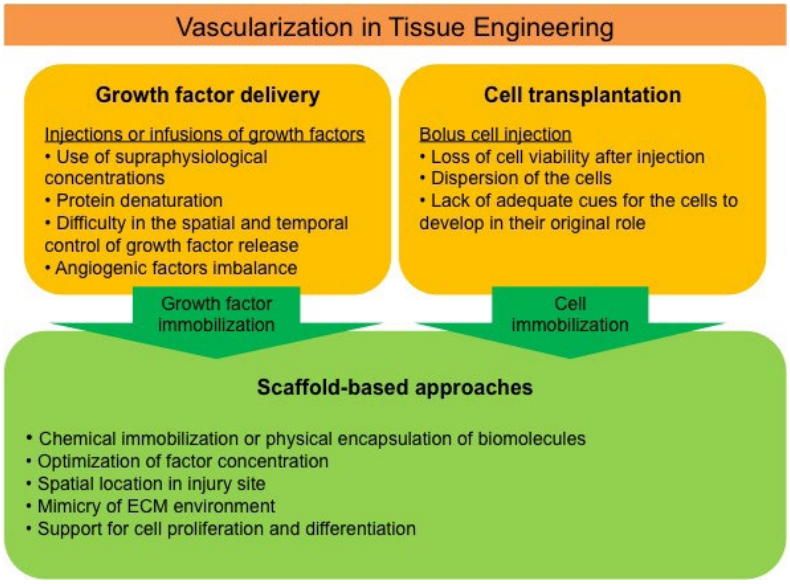

Fig. 1. Summary of strategies used to promote vascularisation in tissue engineering applications. Growth factor delivery and cell transplantation are traditional strategies that present several associated problems and have been improved through their combination and the use of scaffold materials.

review will discuss the usefulness of cellular therapies as a strategy, focusing on the relevance of endothelial progenitor cells as a promising source of endothelial cells **(**ECs**)**, as well as on the importance of co-culture systems and the need to increase their complexity to promote vascularisation in tissue engineering (Fig. 1).

The Vascular System

The vascular network is responsible not only for the transport of gases, nutrients and metabolites, but also for the circulation of signalling molecules, hormones, and antibodies between tissues and organs. The complex structure of the vascular network is achieved by the maturation of the immature blood vessels previously formed by angiogenesis or vasculogenesis (Jain, 2003). Vasculogenesis refers to the *in situ* formation of vessels orchestrated directly by endothelial precursor cells or angioblasts, which is of major importance during embryonic development, but also plays a role in adulthood, both under physiological as well as under pathological conditions (Asahara *et al.*, 1999; Conway *et al.*, 2001). On the other hand, angiogenesis is defined as the process of new blood vessel formation through the sprouting of pre-existing vasculature. For this, specific proteases initiate the degradation of the extracellular matrix (ECM), enabling endothelial cells (ECs) to change polarisation, proliferate and invade the ECM towards the avascular tissue (Costa *et al.*, 2007; Soares, 2009). As ECM is degraded, ECs migrate to distant sites, anastomose and acquire lumens, forming the neovessel, which is then stabilised by the assembly of a basement membrane and adhesion of pericytes. Other stromal cells, such as fibroblasts, also contribute to the formation of the neovessel by releasing growth factors and ECM components. In contrast, vasculogenic processes require circulating endothelial progenitor cells (EPCs) to be stimulated to differentiate into functional ECs that adhere to a vascular bud, assembling into the newly formed vessel (Costa *et al*., 2007; Soares, 2009). Similar to angiogenesis, this process ends by the attachment of mural cells that stabilise the vessel.

Therefore, angiogenesis plays a crucial role throughout postnatal life, and is related to wound healing and the menstrual cycle (Soares, 2009), inflammatory processes, and also some pathological conditions, such as rheumatoid arthritis, diabetic retinopathy, macular degeneration and tumour growth (Costa *et al*., 2007; George *et al.*, 2011). Furthermore, angiogenesis accompanies growth repair, tissue remodelling and regeneration (Carmeliet and Jain, 2000; Jain, 2003; Carmeliet, 2005; Carmeliet and Jain, 2011). The recruitment of new blood vessels through the activation of these two main processes of vascularisation is still considered to be a great challenge in regenerative therapies.

Different blood vessels exist, including arteries, veins and small calibre vessels (arterioles, venules and capillaries) that express characteristic molecular markers. Endothelial precursors that form arteries express ephrin B2 (Efnb2), whereas its receptor, the B4 ephrin receptor (Ephb4), is preferentially expressed in veins (Lin *et al.*, 2007). However, all blood vessels are formed by one thin inner layer of ECs, the endothelium, which is then covered by supporting/mural cells. This vascular endothelium provides a selective barrier, separating the blood stream from the underlying tissues, with vascular wall cells being thus embedded in an extracellular matrix (Fig. 2).

Vascular Wall Cells

Endothelial cells and endothelial progenitor cell populations

Endothelial cells, forming the endothelium, play a pivotal role in vascularisation. They characteristically express von Willebrand factor (vWF), platelet endothelial cell adhesion molecule-1 (PECAM-1 or CD31), and vascular endothelial (VE)-cadherin (Muller *et al.*, 2002; Fuchs *et al.*, 2006a; Fuchs *et al.*, 2006b; Medina *et al.*, 2010a). To maintain the integrity of the endothelium, ECs communicate with

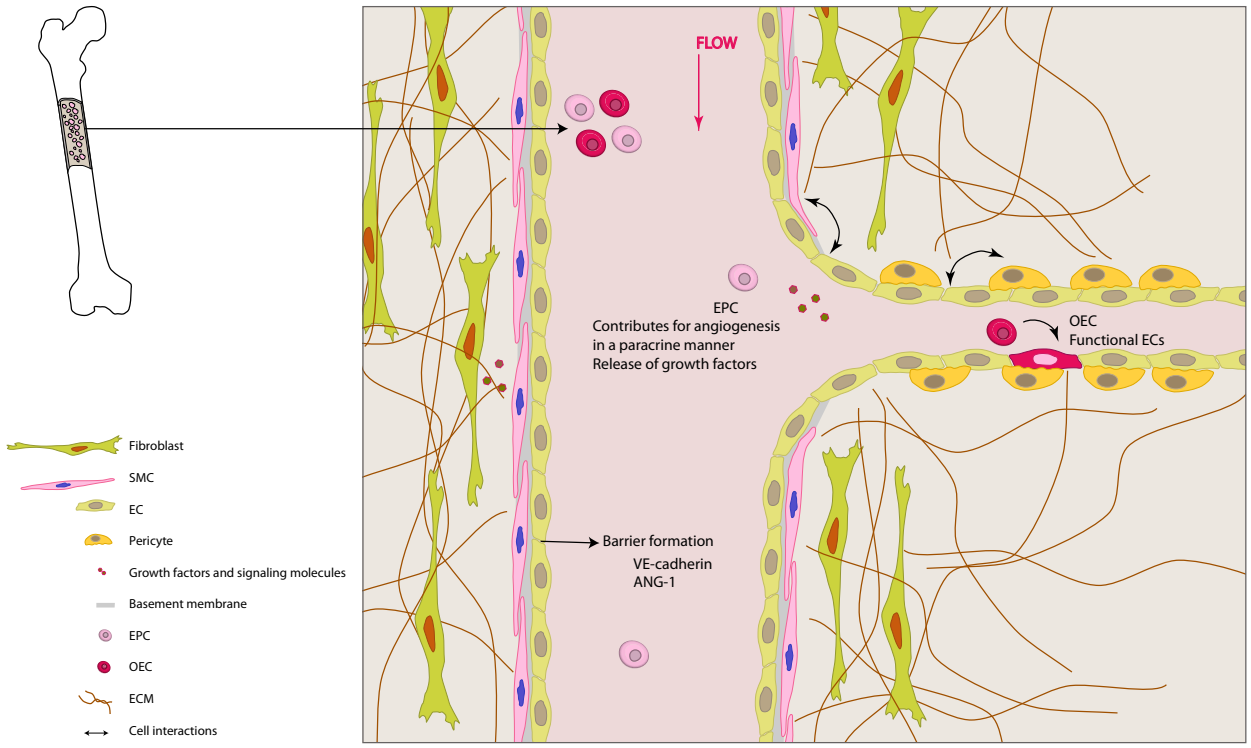

Fig. 2. Schematic representation of the biological processes involved in vascularisation, including cell-cell/ cell-matrix interactions. The vascular endothelium is formed by one layer of endothelial cells (EC). Their expression of adhesion molecules, like VE-cadherin and Ang-1, allows for intercellular interactions, which are important for vessel stabilisation. Vascular maintenance is achieved through specific signalling (release of growth factors and signalling molecules) and direct physical contact between endothelial cells and mural cells, such as smooth muscle cells (SMC) and pericytes, or fibroblasts. The extracellular matrix (ECM) will act as a bridge between blood vessels and the surrounding tissue. Moreover, different populations of endothelial progenitor cells exist in circulation – early endothelial progenitor cells (EPC) and outgrowth endothelial cells (OEC). These cells have distinct roles in vascularisation: EPCs contribute to vascularisation/angiogenesis in a paracrine manner through the release of growth factors, while OECs are able to contribute for the repair and formation of blood vessels by differentiating into mature ECs.

each other through adhesion molecules, like VE-cadherin (Vestweber, 2008), present in adherence junctions, and tight junctions, among other structures (Dejana *et al.*, 1995; Liebner *et al.*, 2006), as well as with neighbouring cells. For instance, connexin (Cx)43 and Cx45 are involved in heterotypic communication with mesenchymal stem cells (MSCs), influencing their differentiation (Villars *et al.*, 2002; Hirschi *et al.*, 2003; Fang *et al.*, 2013).

However, molecular differences exist between endothelial cell populations due to the heterogeneity along this cell lineage (Craig *et al.*, 1998), resulting in high variability when their functional behaviour is assessed through angiogenesis assays, as discussed elsewhere (Staton *et al.*, 2009). Some authors consider that mature ECs present a limited regenerative potential (Sieveking *et al.*, 2008). The discovery of putative endothelial progenitor cells (EPCs) in 1997 (Asahara *et al.*, 1997) contributed to enlarge the knowledge about endothelial cell populations and to change the, until then, prevailing dogma. This stated that throughout postnatal life, new blood vessels could only be generated by fully differentiated ECs. In fact, blood vessels are believed to originate from endothelial precursors (Carmeliet, 2005). Another important characteristic of ECs that contributes to tissue engineering is their plasticity and, consequently, their ability to dedifferentiate into stem cells when stimulated to undergo endothelial-mesenchymal transition, which makes them a potential cell source for use in regenerative strategies, as reviewed elsewhere (Susienka and Medici, 2013).

Although embryonic and adult stem cells exhibit a higher proliferative capacity (Rafii and Lyden, 2003), the use of EPCs emerged as an alternative (Ankeny *et al.*, 2012). EPCs exist in circulation and have been described as being capable of incorporating into vessels after mobilisation from bone marrow, in order to participate in neovascularisation at sites of ischaemia (Asahara *et al.*, 1997). Using these cells overcomes some ethical considerations, along with a deficient knowledge considering the control of stem cell differentiation (Melero-Martin *et al.*, 2007). Two different types of EPCs are believed to exist, early EPCs and late EPCs, here called outgrowth endothelial cells (OECs) (Lin *et al.*, 2000; Sieveking *et al.*, 2008; Medina *et al.*, 2010a; Oshima-Sudo *et al.*, 2011); these cells are commonly isolated from peripheral or umbilical cord blood (Asahara *et al.*, 1997; Ingram *et al.*, 2004; Fuchs *et al.*, 2006a; Fuchs *et*

al., 2006b; Au *et al*., 2008a). Other sources of endothelial progenitors include bone marrow (BM) (Shi *et al*., 1998; Asahara *et al*., 1999; Nolan *et al.*, 2007; Chen *et al.*, 2012), amniotic fluid (Zhang *et al.*, 2009) and adipose tissue (Lin *et al.*, 2008; Szöke *et al.*, 2012). At the same time, new hypotheses about the origin of OECs have emerged in the literature. Tura *et al*. reported that OECs cannot be isolated from bone marrow nor from cells mobilised from this site (Tura *et al.*, 2013). Accordingly, Zhang *et al*. demonstrated that cells isolated from bone marrow with high proliferation during long-term culture were consistently mesenchymal stromal cells and did not differentiate toward the endothelial lineage when cultured under endothelium- promoting conditions (Zhang *et al.*, 2007). This finding defies the concept of BM-derived circulating precursors of endothelial cells, giving rise to further speculation about the possible existence of a vascular source outside the bone marrow. Nevertheless, further studies are needed to confirm this hypothesis.

Despite their origin, these progenitor cells seem to be mobilised into the circulation, contributing to the neovascularisation process (Asahara *et al*., 1997; Asahara *et al*., 1999; Rafii and Lyden, 2003). However, there is still great controversy associated with the term "endothelial progenitor cell" or the cell type that might be preferred for a therapeutic application (Fuchs *et al.*, 2010). Indeed, key issues remain unanswered, like the lack of uniform cellular definitions, and the inadequate functional characterisation (Sieveking *et al*., 2008; Medina *et al.*, 2010b; Oshima-Sudo *et al*., 2011). Early EPCs have been described as BM-derived cells sharing surface markers expressed by haematopoietic stem cell populations, such as CD14, CD45 and CD133 (Asahara *et al*., 1997; Masuda and Asahara, 2003; Medina *et al*., 2010a; Doulatov *et al.*, 2012), differentiating into phagocytic macrophages and possessing myeloid progenitor cell activity (Yoder *et al.*, 2007; Shi *et al.*, 2014). These cells appear after 4 to 7 days in culture and are thought to provide angiogenic factors, acting in a paracrine manner, whereas OECs appear much later, after 14 to 21 days, exhibiting typical endothelial characteristics and being reported to incorporate into resident vasculature (Mukai *et al.*, 2008; Sieveking *et al*., 2008; Oshima-Sudo *et al*., 2011). Moreover, early EPCs have been shown to preferentially express genes involved in immune responses and inflammation, while OECs express genes involved in development and angiogenesis, including the angiopoietin receptor Tie2, endothelial nitric oxide synthase (eNOS), ephrins and transforming growth factor-β (TGF-β) (Cheng *et al.*, 2013; Medina *et al*., 2010a). A more recent study also showed that umbilical cord blood (UCB)-derived OECs secrete a broad spectrum of pro-inflammatory and angiogenic cytokines, including angiogenin, angiopoietin (Ang)-2 and platelet-derived growth factor (PDGF)-BB (Liu *et al.*, 2012), one of the four homodimeric glycoproteins from PDGF family.

In addition, mature ECs are also capable of producing ECM components (Hurley *et al.*, 2010). Similarly, OECs have been reported to deposit collagen IV, laminin and fibronectin with an increasing organisation over time (Kusuma *et al.*, 2012), which is essential for EC assembly.

Mural cells

Mural cells are recruited to allow vessel maturation and stability (Jain, 2003). ECs first associate to form tubules and afterwards different signals are involved in the recruitment and coverage of ECs by mural cells, including platelet-derived growth factor (PDGF)-B, Ang-1, TGF-β and NOTCH signalling (Carmeliet and Jain, 2011).

Different mural cells exist depending on their morphology, location and the expression of specific markers, being divided into pericytes and smooth muscle cells (SMCs) (Gaengel *et al.*, 2009). Vascular SMCs are present in larger blood vessels, such as arteries and veins, forming multiple concentric layers, being embedded within the vascular basement membrane and, consequently, separated from the endothelium. On the other hand, pericytes cover capillaries and other microvessels, being often organised in a discontinuous cell layer around the endothelial cell tube (Gaengel *et al*., 2009). Newly formed vasculature would regress without the support of these peri-endothelial cells (Conway *et al*., 2001), which contribute to stabilise blood vessels through direct physical contact (Bergers and Song, 2005), ECM deposition (Njauw *et al.*, 2008) and growth factors release, as vascular endothelial growth factor (VEGF) and Ang-1 (Bergers and Song, 2005; Njauw *et al*., 2008; Carmeliet and Jain, 2011).

Mural cells not only stabilise blood vessels by inhibiting EC proliferation and migration, but are also involved in the modulation of blood flow and vessel permeability.

Different tissues exhibit varying mural cell phenotypes and different degrees by which they cover the endothelium (Gaengel *et al*., 2009); thus, one cell type might be better suited – depending on the application of the vascularised engineered tissue construct.

Fibroblasts

Fibroblasts are mesenchymal cells, and can be quite different in terms of cell dynamics, depending on their tissue of origin (Chang *et al.*, 2002). They are the main source of ECM components (Chang *et al*., 2002; Kalluri and Zeisberg, 2006), including collagen I, fibronectin and proteoglycans (Mansbridge *et al.*, 1999). Human fibroblasts are abundant in the dermis and can be easily obtained from minimally invasive skin biopsies, using standard laboratory protocols (Wong *et al.*, 2007; Junker *et al.*, 2013). Consequently, fibroblasts were the first cells from which induced pluripotent stem cells (iPSCs) were obtained, first from mice (Takahashi and Yamanaka, 2006) and then from human origin (Takahashi *et al.*, 2007). Sir John B. Gurdon and Shinya Yamanaka were awarded the 2012 Nobel Prize in Physiology or Medicine for their research in the area of reprogramming mature cells to become pluripotent (Yamanaka, 2013). Since the discovery of iPSCs, human fibroblasts have been reprogrammed to become endothelial cells in response to defined media and culture conditions, being able to form capillary-like structures *in vivo* in a Matrigel plug mice model (Margariti *et al.*, 2012). Therefore, human dermal fibroblasts have been much used for skin tissue engineering applications and have gained an increasing interest as a cell source for other applications, like neovascularisation. For instance, the adventitial layer of large blood vessels

is mainly composed of fibroblasts and associated ECM components (Njauw *et al*., 2008). Fibroblasts secrete potent angiogenic factors, like VEGF and fibroblast growth factor (FGF)-2, as well as matrix metalloproteinase (MMP)-2 and MMP-9 (Berthod *et al.*, 2006), which in turn act by releasing pro-angiogenic peptides. Hence, it is possible that fibroblasts act as peri-endothelial progenitors *in vivo* (Njauw *et al*., 2008), supporting EC survival and migration, as well as modulating the expansion of capillary-like networks, particularly *in vitro* (Kunz-Schughart *et al.*, 2006). Moreover, since fibroblasts generate a scaffold for other cells through matrix deposition, they may alter the mechanical extracellular microenvironment, thus regulating the vascularisation processes (Hurley *et al*., 2010).

The extracellular matrix

The ECM acts as a bridge between vascular wall cells and the respective surrounding tissues. Providing a 3D support for EC proliferation and survival, the ECM has a dual role – it acts as an adequate substrate for the organisation of ECs into microvessels, simultaneously retaining and concentrating growth factors in the cellular microenvironment (Berthod *et al*., 2006). ECM is therefore considered a reservoir of proteins that are involved in several physiological events, including wound healing and angiogenesis. The presence of proteases or protease inhibitors leads to changes in the bioavailability of matrix-sequestered factors (Sottile, 2004).

Capillary morphogenesis is regulated through the immobilisation of angiogenic cytokines, growth factors and other molecular cues involved in angiogenic activation (*e.g.*, collagen I) (Davis and Senger, 2005), as well as by angiogenesis inhibitors (Hynes, 2007). MMPs exist, which release pro-angiogenic (Demidova-Rice *et al.*, 2011) and anti-angiogenic peptides (Brauer *et al.*, 2011), through ECM cleavage. In particular, the endothelium is separated from the connective tissue by the basement membrane, a specialised layer of ECM where collagen IV and laminin organise into networks, being both essential for basement membrane stability (LeBleu *et al.*, 2007). For instance, collagen IV has been described as having the capacity to modulate the angiogenic response in a rat aorta model, resulting in the elongation of the neovessels (Bonanno *et al.*, 2000). On the other hand, cleavage of collagen IV may result in the release of tumstatin and other angiogenesis inhibitors (Hynes, 2007).

Given that ECM proteins also possess binding sites for cells to adhere to through their surface receptors, ECM acts as a key controller of cell behaviour, activating several intracellular signalling pathways and, in turn, cells degrade and remodel the ECM (Davis and Senger, 2005; LeBleu *et al*., 2007; Daley *et al.*, 2008). The ECM corresponds, thus, to a natural scaffold for tissue development and repair, supporting tissue reconstruction (Badylak, 2002). Hence, understanding cell-matrix interactions is of major importance to mimic the natural ECM when developing functionalised artificial matrices (Munarin *et al.*, 2011).

Cellular therapies to promote vascularisation

Despite the great advances in the field of tissue regeneration, the vascularisation of an implanted scaffold is still a critical aspect. Two approaches have emerged aiming at solving this problem (Fig. 3). One is focused on culturing cells into the scaffold and implanting the system without any cell organisation, while the other consists of pre-vascularising the system prior to implantation. However, it is not known which strategy would lead to a better functional anastomosis between vessels formed in the scaffold and the host vasculature.

Transplantation of endothelial populations

Cell transplantation, particularly of ECs, has been the focus of several strategies aiming at promoting vascularisation. Endothelial cells, particularly progenitor cells, have been widely investigated to treat cardiovascular diseases. Provided that progenitor cell populations (both EPCs and OECs) can be easily isolated from blood, their use in an autologous way constitutes a major advantage for clinical applications. Clinical trials concerning the transplantation of both UCB-derived progenitor cells and BM-derived mononuclear cells have proved the potential of these cells for the treatment of cardiovascular diseases, since significant beneficial effects on left ventricle remodelling processes were observed in patients with acute myocardial infarction (TOPCARE-AMI trial) after cell transplantation (Assmus *et al.*, 2002; Britten *et al.*, 2003; Schächinger *et al.*, 2004).

In addition, the use of EPCs in animal models has been proven efficient concerning revascularisation of injured tissues. In general, the delivery of EPCs, particularly through hydrogels, has been reported to promote systemic and renal protective effects in mice during the treatment of acute kidney injury conditions, such as induced nephropathy, endotoxaemia and sepsis, leading to an improvement of microvascular competence, as reviewed elsewhere (Ratlif and Goligorsky, 2013). Endothelial cells have also been used to treat other ischaemic lesions. Exogenous administration of human peripheral blood (PB)-derived EPCs in a murine model of hind limb ischaemia resulted in an improvement of vascularisation, together with blood flow recovery and a reduction in limb necrosis and auto-amputation (Kalka *et al.*, 2000). Silva and colleagues (2008) implanted human UCB-derived EPCs and OECs together using an artificial ECM as a scaffold (modified alginate) in a peripheral artery disease mouse model and showed that this co-transplantation approach provided a great benefit for the neovascularisation of ischaemic muscle tissue, compared to the delivery of each cell population separately (Silva *et al.*, 2008). This study demonstrated that the co-transplantation of cell populations with complementary functions might result in a broad benefit in vascularisation strategies and revealed that the utility of EPCs highly depends on the delivery mode, as well as on the control over cell fate after transplantation.

Vascularisation of 3D tissue engineered structures strongly depends on cell viability. When ECs are cultured alone, their survival seems to be impaired. Studies reporting

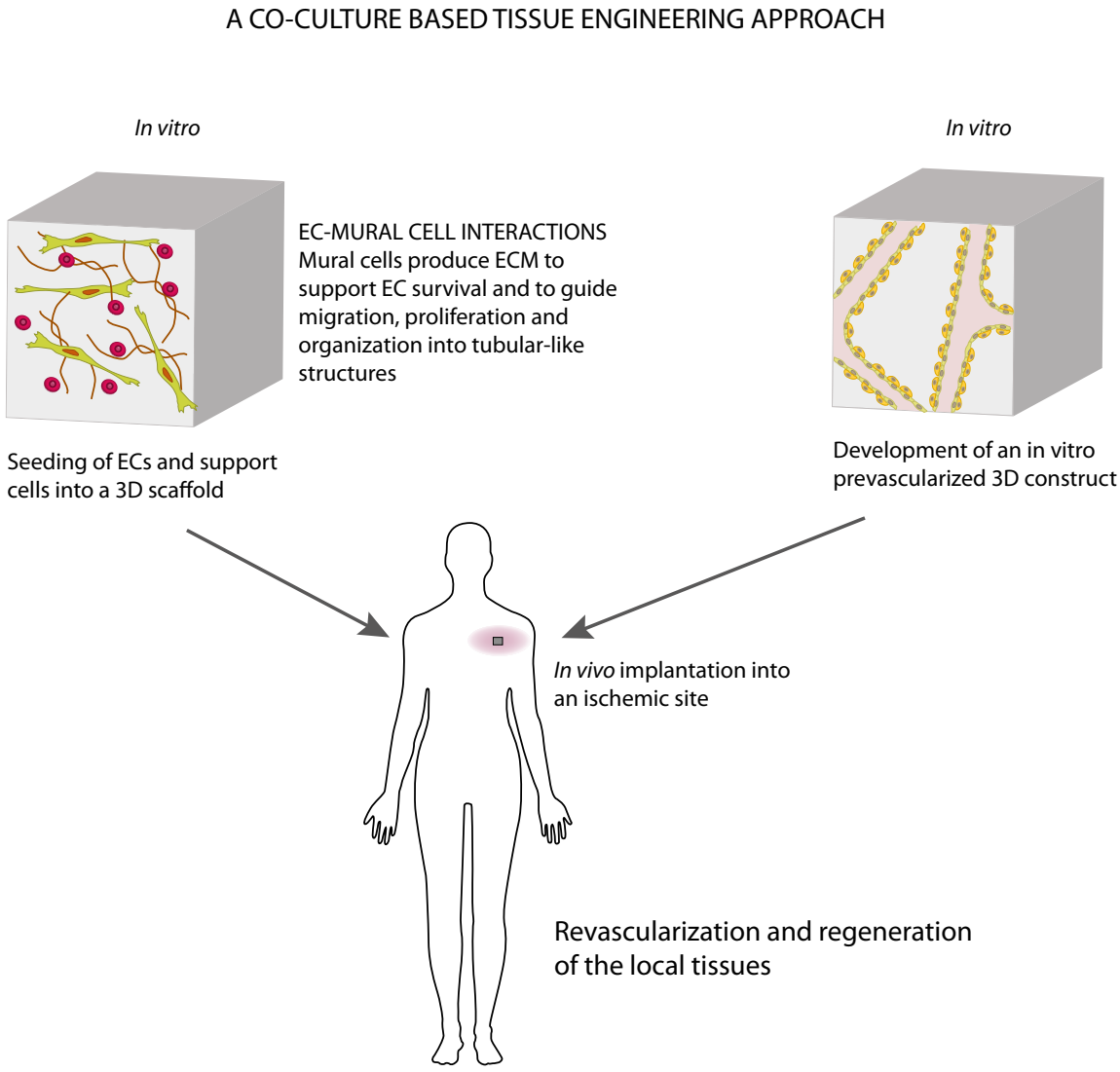

Fig. 3. A co-culture-based tissue engineering approach for the improvement of revascularisation and regeneration of ischaemic tissues. One strategy consists of seeding endothelial cells (EC) and mural cells in a 3D scaffold followed by *in vivo* implantation. Another strategy involves the development of a prevascularised 3D scaffold before *in vivo* implantation.

the presence of cells that express α-smooth muscle actin (α-SMA), a phenotypic marker of myofibroblasts and pericytes, around blood vessels, strongly suggest that formation of a stable vascular network by ECs is highly dependent on their co-implantation with supporting perivascular cells (Melero-Martin *et al.*, 2007; Njauw *et al.*, 2008; Finkenzeller *et al.*, 2009; Chen *et al.*, 2010; Dohle *et al.*, 2011; Berthod *et al.*, 2012).

Co-culture systems

The importance of a co-culture system relies on the intricate communication pathways that are established between different cell types, both through diffusible signalling molecules and by cell-cell contacts, with angiogenesis being controlled by the interaction between ECs and other cell types (Fuchs *et al.*, 2007). Hence, this section will focus on the use of ECs in co-culture systems to promote vascularisation in tissue engineering. Table 1 summarises the state-of-the-art literature regarding 2D and 3D co-culture systems for tissue engineering purposes.

Co-cultures of ECs with pericytes

Pericytes have been recently described as $CD146^{+}$ and $CD34^{-}$ cells; however, these markers are not reliable *in vitro* and functional assays are needed (Blocki *et al.*, 2013). Nevertheless, pericytes were considered as a multipotent population, sharing commonly accepted MSC markers and features, as discussed elsewhere (Chen *et al.*, 2009a). Recent advances have suggested that pericytes might include multilineage progenitors (Crisan *et al.*, 2008) and that the walls of blood vessels can possibly be a source of these stem/progenitor cells (Corselli *et al.*, 2012).

Pericytes have been investigated in co-culture systems with endothelial cells to mimic the blood brain barrier (Hayashi *et al.*, 2004; Zozulya *et al.*, 2008), or the gliovascular complex from the neurovascular unit (Itoh *et al.*, 2011), as well as the retinal microvascular environment to study diabetic retinopathy (Tarallo *et al.*, 2012). Although pericytes are described as being capable of protecting the blood brain barrier from disruption, for instance, following short periods of hypoxia (Hayashi *et*

Table 1. Examples of co-cultures of endothelial cells and support cells.

2D or 3D culture	*In vitro* or *in vivo*	Cell types used	Main results	References
2D	*In vitro*	• Pericytes • HUVECs	In co-cultures, ECs stimulated the activation of pericytes, which produced VEGF, leading to EC organisation into capillary-like structures after 24 h.	(Kale *et al.*, 2005)
	In vitro	• MSCs • ECs (HDMECs, OECs)	Increased release of angiogenic growth factor by MSCs and overexpression of $VEGF_{165}$ were observed. α-SMA expressing cells appeared around capillary-like structures.	(Kolbe *et al.*, 2011; Laranjeira *et al.*, 2012)
	In vitro	• Osteoprogenitor cells • ECs (HUVECs, HDMECs, OECs)	ECs expressed BMP-2 and BMP-4 and increased MSCs differentiation, together with a higher angiogenic activity. Increased expression of osteogenic differentiation markers have been detected.	(Bidarra *et al.*, 2011; Grellier *et al.*, 2009a; Guillotin *et al.*, 2008; Kaigler *et al.*, 2005; Smadja *et al.*, 2008)
3D	*In vivo*	• Pericytes (derived from 10T1/2) • EPCs	In co-implantation approaches, EPCs aligned into functional blood vessels.	(Au *et al.*, 2008a)
	In vitro/ In vivo	• Pericytes • HUVECs	Pericytes supported the assembly of ECs into capillary-like structures in 3D Matrigel assay/ Improved angiogenesis in a myocardial infarction mouse model.	(Chen *et al.*, 2013)
	In vivo	• HUASMCs • HUVECs	The implantation of a decellularised small intestinal mucosa scaffold led to a rapid vascularisation of the graft.	(Wang *et al.*, 2012)
	In vivo	• SMCs • OECs	Co-implantation resulted in a higher microvascular density than that of OECs alone. Functional anastomoses with the host vasculature have been observed.	(Foubert *et al.*, 2008; Melero-Martin *et al.*, 2007)
	In vitro	• Fibroblasts • HUVECs	Fibroblast-derived matrix was essential for lumen formation by ECs. α-SMA expressing cells appeared around capillary-like structures. Fibroblasts supported the assembly of ECs into capillary-like structures.	(Berthod *et al.*, 2006; Berthod *et al.*, 2012; Griffith *et al.*, 2005; Guerreiro *et al.*, 2013; Kunz-Schughart *et al.*, 2006; Newman *et al.*, 2011)
	In vivo	• Fibroblasts • OECs	In a prevascularisation approach, blood perfusion and anastomosis with the host vasculature occurred faster (within 27 h). OECs accelerated wound healing by incorporating into newly formed blood vessels and promoting oxygenation.	(Chen *et al.*, 2010; Hendrickx *et al.*, 2010)
	In vivo	• MSCs • HUVECs	MSCs differentiated into pericytes and stabilised the newly formed vasculature for more than 130 days.	(Au *et al.*, 2008b)
	In vitro	• MSCs • ECs (progenitor-derived)	An increased mRNA expression of junctional proteins (connexin 43) was observed.	(Guerrero *et al.*, 2013)
	In vitro	• MSCs • EPCs	MSCs influenced the differentiation of EPCs towards an endothelial functional phenotype and the formation of capillary-like structures was observed after 7 days. EPCs influenced the differentiation of MSCs towards a pericyte-like phenotype.	(Duttenhoefer *et al.*, 2013; Loibl *et al.*, 2014)
	In vivo	• HBMSCs • ECs (HDMECs, HUVECs)	ECs functioned to form vasculature and to promote osteogenic differentiation. Significant mineralisation has been observed in a bone defect.	(Grellier *et al.*, 2009b; Kaigler *et al.*, 2005)
	In vivo	• Osteoblasts/ osteoprogenitors • OECs	Co-implantation resulted in a rapid formation of OEC-derived vessels, which were shown to be functional (i.e. anastomosed with the host vasculature and were functional).	(Fuchs *et al.*, 2009; Ghanaati *et al.*, 2011)
	In vitro	• Hepatocytes • ECs	ECs supported the maintenance of hepatocyte-specific functions, while organising into capillary-like structures.	(Salerno *et al.*, 2011; Shang *et al.*, 2014)
	In vitro	• Cardiomyocytes • HUVECs	ECs organised into capillary-like structures and contributed for the survival and contractility of cardiac cells.	(Garzoni *et al.*, 2009)

al., 2004), studies in the field of vascularisation are still scarce. Some authors developed *in vitro* models of the gliovascular complex and reported that capillary-like structures are maintained over time, due to the presence of pericytes in co-cultures of endothelial cells and astrocytes (Itoh *et al.*, 2011), but pericytes are not the focus of these research models.

On the other hand, the 10T1/2 cell system, a mouse embryonic cell line, has been used as a precursor of pericytes or SMCs, since their differentiation can be induced by TGF-β, produced by human umbilical vein endothelial cells (HUVECs) in a co-culture system (Kale *et al.*, 2005). In turn, pericytes were shown to produce VEGF, stimulating ECs to organise into capillary-like structures, with markers of activated pericytes, like aminopeptidase N, being detected after the first 24 h of culture (Kale *et al.*, 2005).

The same pericyte precursors were also used by Au and colleagues (2008) to investigate whether the implantation of higher cell densities of UCB-derived EPCs could obviate the need for a co-implantation approach (Au *et al.*, 2008a). Although some of the implanted EPCs aligned into blood vessels, these vessels were only transiently perfused and regressed after 23 days, with 10T1/2 cells functioning as perivascular cells *in vivo* (Au *et al.*, 2008a).

Recently, human pericytes started to be used – aiming at tissue engineering applications. Human microvascular pericytes, isolated from skeletal muscle biopsies, have been shown to improve cardiac function of ischaemic heart after intra-myocardial injection, being able to promote host

angiogenesis both in the peri-infarct areas and within the blood-deprived infarct region (Chen *et al.*, 2013). The same study also demonstrated that pericytes support HUVECs in the formation of capillary-like structures in an *in vitro* 3D Matrigel assay (Chen *et al*., 2013).

Despite the fact that vascularisation starts with capillaries and progressively increases complexity in terms of cell organisation, co-culture models have been focusing on cells that are mainly present in large vessels, instead of aiming to understand the role of pericytes in supporting vascularisation. Nevertheless, pericytes have been applied in implantation strategies using immune-competent mouse models and no inflammatory response was detected (He *et al.*, 2010). Although further studies are needed, this immunosuppressive function of pericytes might be favourable for future clinical use of these cells, if they are of heterologous source.

Co-cultures of ECs with smooth muscle cells

SMCs are involved in the stabilisation and maturation of blood vessels. A recent study has shown that implantation of a decellularised porcine small intestinal mucosa scaffold reseeded with HUVECs and human umbilical artery SMCs (HUASMCs) resulted in a rapid vascularisation (4 days) of the graft, together with the maintenance of favourable mechanical properties of the tissue engineered vascular grafts (Wang *et al.*, 2012).

Co-administration of ECs and SMCs has also been investigated in models of ischaemia. For instance, Foubert *et al*. implanted endothelial and smooth muscle progenitor cells, both isolated from human UCB (OECs and SMPCs, respectively), in combination into an ischaemic leg of nude mice and verified that this combined administration resulted in a higher microvascular density, compared to OECs implanted alone (Foubert *et al.*, 2008). In addition, molecular events that are involved in vascularisation steps also appear to be different in co-culture systems, compared to ECs cultured alone. For instance, PDGF-B was found to be down-regulated over time in co-culture spheroids of HUVECs/HUASMCs, contrasting to HUVECs alone (Korff *et al.*, 2001). This suggests that co-cultures properly mimic the *in vivo* phenotype (Korff *et al*., 2001), since the expression of PDGF-B is restricted to immature capillaries. Besides, Foubert *et al*. have demonstrated that SMCs released Ang-1 and its receptor Tie-2 was subsequently activated in OECs, showing that paracrine release of Ang-1 modulated OEC incorporation into the vascular endothelium (Foubert *et al*., 2008).

In addition, Melero-Martin and colleagues first demonstrated the *in vivo* vasculogenic potential of OECs by performing a co-implantation protocol of both human UCB- or PB-derived OECs together with SMCs in Matrigel plugs through subcutaneous injection into immunodeficient mice. This approach resulted in the formation of human EC-lined vessels, which contained murine erythrocytes, with functional anastomoses being observed (Melero-Martin *et al*., 2007). They have shown that blood vessel formation occurred only for co-implantation experiments (Melero-Martin *et al*., 2007), reinforcing the importance of cellular crosstalk.

Co-cultures of ECs with fibroblasts

In the past decade, different works have focused on the potential of fibroblasts to enhance vascularisation. The implantation of neonatal human dermal fibroblasts using Matrigel plugs in mice showed that fibroblasts induced the ingrowth of blood vessels from the host vasculature (Guerreiro *et al.*, 2012), thus suggesting that these cells take part in the recruitment of ECs *in vivo*. Moreover, a combination of fibroblast-derived proteins, comprising Ang-1, angiogenin, hepatocyte growth factor (HGF), TGF-α and tumour necrosis factor (TNF), has been described to support EC sprouting, while matrix proteins, such as collagen I, secreted protein acidic and rich in cysteine (SPARC) and insulin-like growth factor-binding protein 7 (IGFBP7), among other factors, were necessary for lumen formation by HUVECs co-cultured with normal human lung fibroblasts in 3D fibrin gels, which proved to be related to an increased matrix stiffness (Newman *et al.*, 2011). In particular, when ECM synthesis by fibroblasts is reduced, EC tube formation is decreased (Berthod *et al*., 2006). However, ECM synthesis seems not sufficient for the organisation of capillary-like structures, which requires a close association between ECs and living fibroblasts (Berthod *et al*., 2006). It has also been described that pericytes spontaneously originate from fibroblasts, when co-culture systems with ECs are established (Berthod *et al*., 2012). In addition, a spheroid co-culture model has demonstrated that HUVECs were capable of attaching and migrating along human foreskin fibroblasts-derived ECM in order to form a capillary-like network (Kunz-Schughart *et al*., 2006). Griffith *et al*. reported a rapid sprouting of HUVECs seeded on microcarrier beads and co-cultured with human dermal fibroblasts, with capillary-like networks forming in a fibrin-based tissue construct after 2-3 days and continuing to remodel up to 14 days (Griffith *et al.*, 2005). Guerreiro *et al*. also demonstrated that when neonatal human dermal fibroblasts were immobilised in alginate gels grafted with an RGD peptide sequence and co-cultured with HUVECs, fibroblasts had the ability to modulate and support the assembly of ECs into capillary-like structures (Guerreiro *et al.*, 2013). In addition, the simultaneous use of bioactive silicate materials together with co-cultures of human dermal fibroblasts and HUVECs resulted in an enhanced formation of highly anastomosed capillary-like structures, with calcium silicate extracts inducing VEGF expression by fibroblasts (Li and Chang, 2013). Moreover, normal human lung fibroblasts have been co-cultured with HUVECs in a 3D ECM construct, which allowed the generation of *in vitro* perfusable microvessel networks, through the use of microfluidic technology (Kim *et al.*, 2013).

The implantation of fibroblasts in co-culture with OECs using 3D tissue constructs has also been described. OECs have been reported to accelerate wound healing for skin applications. PB-derived OECs integrated into human dermal fibroblast layers were capable of actively incorporating into new blood vessels, promoting re-oxygenation of the wound bed (Hendrickx *et al.*, 2010).

Chen and colleagues created prevascularised tissue constructs by mixing HUVECs and normal human lung fibroblasts in a fibrinogen solution polymerised through the

addition of thrombin, in a proof of concept approach (Chen *et al.*, 2009b). Then, human UCB-derived OECs were used and, after being organised into capillary-like networks *in vitro*, fibrin-based tissue constructs were implanted. Blood perfusion and the formation of anastomosis between vessels from the implanted structure and the host vasculature were observed within 27 h after implantation, when OECs were co-cultured with a high density of fibroblasts (Chen *et al.*, 2010).

Considering these data together, further studies should be performed using co-cultures of OECs and fibroblasts, in order to understand the molecular mechanisms underlying this cellular crosstalk during the angio-/vasculogenic processes.

Co-cultures of ECs with mesenchymal stem cells

MSCs can be obtained not only from bone marrow, but also from a variety of other tissues, including blood, adipose tissue, muscle and dermis (Singer and Caplan, 2011). Adult MSCs have gained an increasing interest for regenerative therapies, due to their ability to differentiate into bone, cartilage and fat, together with their immunomodulatory functions (Pittenger *et al.*, 1999; Haniffa *et al.*, 2009; Singer and Caplan, 2011). On the other hand, some MSCs may differentiate into SMCs after implantation, suggesting that these cells may function as perivascular cells (Au *et al.*, 2008b). MSCs are multipotent cells and, thus, overcome the problems of using terminally differentiated cells, including the long fabrication time, given their low proliferative capacity and the lack of off-shelf capacity of engineered constructs (He *et al.*, 2010). Au *et al.* have demonstrated that human BM-derived MSCs were capable of differentiating into functional perivascular cells when co-implanted with HUVECs, stabilising nascent blood vessels, which remained functional for more than 130 days *in vivo* (Au *et al.*, 2008b). This work also showed that MSCs-derived perivascular cells were able to act in a physiological way, by constricting in response to the addition of endothelin-1, a peptide with endogenous vasoconstrictive function (Au *et al.*, 2008b). Besides, Laranjeira *et al.* performed co-cultures of human dermal microvascular ECs (HDMEC) with human MSCs and observed that collagen I, which is a pro-angiogenic substrate, and $VEGF_{165}$ were overexpressed, compared to monocultures of MSCs (Laranjeira *et al.*, 2012). Guerrero *et al.* showed that there is an increase in the mRNA levels of junctional proteins like Cx43, when progenitor-derived ECs are co-cultured with MSCs, compared to monocultures (Guerrero *et al.*, 2013).

Starting from the hypothesis that the combination of PB-derived OECs and MSCs could result in a beneficial effect for the generation of pre-vascularised tissue engineered constructs, Kolbe and colleagues performed co-cultures of both cell types in order to study their angiogenic behaviour. In contrast to OECs in monoculture, an increased release of angiogenic factors by MSCs and a considerable formation of angiogenic structures were observed in co-cultures, which obviously depended on the culture media used (Kolbe *et al.*, 2011). These microvessel-like structures appeared closely associated with cells that stained positive for SMA (Kolbe *et al.*, 2011). These results highlight the relevance of MSCs as supporting cells for the stabilisation and maturation of newly formed vascular networks, resulting from cellular communication.

Furthermore, the addition of PB-derived EPCs to an *in vitro* pre-vascularisation model of OECs/MSCs significantly improved the formation of capillary-like structures, with EPCs acting as pro-angiogenic myeloid cells and being co-localised with these structures (Shi *et al.*, 2014). Thus, EPCs might play a role in the *in vivo* formation of bioengineered vascular networks (Melero-Martin *et al.*, 2010). BM-derived EPCs ($CD34^+/CD133^+$) have been recently reported as forming capillary-like networks when co-cultured with BM-derived MSCs in a 3D polyurethane scaffold, with luminal tubular structures being observed after 7 days, which did not occur when EPCs were cultured alone (Duttenhoefer *et al.*, 2013). Although EPCs have traditionally been known as participating in angiogenesis through the secretion of paracrine factors, instead of directly incorporating into blood vessels, this work demonstrated the influence of MSCs in the differentiation process towards a mature and functional EC phenotype. Indeed, endothelial markers, including CD31 and vWF were detected in the system (Duttenhoefer *et al.*, 2013), with BM-derived EPCs also influencing the differentiation of MSCs towards a pericyte-like phenotype (Loibl *et al.*, 2014).

Finally, ECs have also been described as potentiating the osteogenic response of human foetal MSCs (hfMSCs) *in vitro, via* paracrine signalling, together with a more robust neovascularisation in co-cultures of OECs/hfMSCs (Liu *et al.*, 2012), showing that co-cultures constitute a bi-directional system.

Co-cultures with osteoblasts

There is already extensive literature regarding the crosstalk between differentiated ECs and osteoblastic cells, which has been widely investigated to evaluate both the angiogenic and the osteogenic potential for bone tissue regeneration (Fuchs *et al.*, 2007; Fuchs *et al.*, 2009a; Fuchs *et al.*, 2009b; Grellier *et al.*, 2009a; Grellier *et al.*, 2009b; Dohle *et al.*, 2010; Ghanaati *et al.*, 2011). A study concerning cell encapsulation within alginate microspheres showed that mature ECs alone presented almost no metabolic activity after 8 days of *in vitro* culture but their co-immobilisation with osteoblasts resulted in an enhanced performance in terms of metabolic activity and survival (Grellier *et al.*, 2009b).

ECs were shown to secrete bone morphogenetic protein (BMP)-2 (Kaigler *et al.*, 2005) and BMP-4 (Smadja *et al.*, 2008), which influence MSC differentiation into osteoblasts (Bidarra *et al.*, 2011) that, in turn, release VEGF, enhancing EC viability (Kaigler *et al.*, 2005; Kaigler *et al.*, 2006; Clarkin *et al.*, 2008; Grellier *et al.*, 2009a; Grellier *et al.*, 2009b). In addition, in co-cultures of primary human osteoprogenitors (HOPs) and HUVECs, mRNA levels of TGF-β1 and VEGF were upregulated in HOPs, together with an increase of alkaline phosphatase (ALP) activity, stimulated by the co-culture (Guillotin *et al.*, 2008).

Grellier *et al.* performed co-cultures of HOPs with HUVECs, and reported the formation of a capillary-like

network after 48 h in culture, with ECs using osteoblasts as a matrix for spreading (Grellier *et al*., 2009a). These mature ECs exhibited a similar organisation when cultured in the presence of conditioned medium from co-cultures, suggesting that a direct contact is needed for the production of chemo-attractive factors, since the same organisation was not observed using conditioned medium from osteoblastic cells cultured alone (Grellier *et al*., 2009a). Moreover, *in vivo* assays showed that human bone marrow stromal cells and HUVECs immobilised within alginate microspheres promoted a significant mineralisation in a bone defect, compared to the marrow cells alone (Grellier *et al*., 2009b), which reinforces the need for intrinsic cellular crosstalk in physiological processes, including bone tissue regeneration.

In general, as previously mentioned, OECs are believed to exhibit a promising angiogenic potential that needs to be further explored and understood. In fact, when co-cultured with osteoblasts, PB-derived OECs are capable of forming pre-vascular networks with a vascular lumen both in 2D cultures and in 3D spheroids, which has not been observed for HUVECs (Fuchs *et al*., 2007). This might be explained by differences in OEC and HUVEC assembly along rigid collagen matrices (Sieminski *et al.*, 2004; Sieminski *et al.*, 2005). On the other hand, Hoffman *et al*. performed co-cultures of HUVECs with primary osteoblasts in polyurethane scaffolds and verified the formation of multiple tube-like structures, with osteoblasts supporting survival, proliferation and *in vitro* vasculogenesis by HUVECs (Hofmann *et al.*, 2008).

Moreover, the same authors also performed a similar co-culture system using starch polycaprolactone fibre meshes. They reported the formation of vascular structures closely associated with the scaffold material, observing an *in vivo* OEC-mediated scaffold vascularisation when OECs were co-implanted with primary osteoblasts, which was not observed for OECs alone (Fuchs *et al*., 2009a). These co-culture techniques seem to induce an angiogenic activation of OECs, resulting in microvessel-like structures for a pre-vascularisation strategy. Although other studies reported no sign of *in vivo* formation of a bone-specific matrix (Ghanaati *et al*., 2011), this beneficial effect of co-cultures could be the result of embedding ECs into a rich ECM produced by the osteoblasts, as well as from an angiogenic activation of ECs through paracrine effects.

In addition, the dynamic development of the same co-culture system in silk fibroin constructs was also evaluated and both an increase and a maturation of the *in vitro* vascular network with culture time was demonstrated through an increase in parameters like tube area, tube length, number of nodes and number of meshes (Fuchs *et al*., 2009b). Moreover, a time scale has been established, over which human functional vessels are formed when co-cultures of OECs and primary osteoblasts are used. By implanting *in vitro*-generated co-cultures into immunodeficient mice they observed that, after 2 days, there was evidence of OEC-specific vessels and that some human-derived microvessels were already anastomosed with the host vasculature and, simultaneously, several perfused vessels were observed (Ghanaati *et al*., 2011).

At the molecular level, when these co-cultures were used, an increase in the expression of osteopontin and osteocalcin, markers of osteoblastic differentiation, as well as an upregulation of ALP and collagen I, has been observed (Fuchs *et al*., 2009b; Grellier *et al*., 2009a). In addition, mRNA expression of $VEGF_{165}$ has been detected in monocultures of osteoblasts both in static 2D and dynamic 3D cultures, while the expression of VEGF receptors, Flt-1 and KDR, was significantly upregulated in co-culture systems (Grellier *et al*., 2009a; Grellier *et al*., 2009b). This confirms that osteoblasts produce $VEGF_{165}$, being the protein immediately used by ECs in angiogenic activation processes. Overall, these experiments indicate an ongoing differentiation of the osteoblastic cells in co-culture with OECs, as well as ECM deposition (Fuchs *et al*., 2009a; Fuchs *et al*., 2009b) and pro-angiogenic factors production by osteoblasts (Dohle *et al*., 2010). Together, these studies demonstrate that co-cultures or co-implantation of ECs with osteoblastic cells result in a beneficial effect concerning vascularisation processes and osteogenic differentiation.

Co-cultures with other specialised cells

Through the years, ECs have been used aiming at engineering complex organs. In the field of liver tissue engineering, besides vascularisation, preserving and maintaining hepatocyte phenotype and function constitutes another challenge (Shang *et al.*, 2014). Layer-based co-culture models have been developed to mimic key features of liver sinusoids. Here, heterotypic contacts between primary hepatocytes and ECs, either rat liver sinusoidal ECs or modified HUVECs, resulted in the maintenance of hepatocyte-specific functions, having, for instance, beneficial effects on urea and albumin production (Kim and Rajagopalan, 2010; Kang *et al.*, 2013; Shang *et al*., 2014). In addition, Salerno and colleagues developed an organotypic co-culture system and reported that hepatocytes exhibited well-demarcated cell-cell borders and organised into compact polyhedral cells with round nuclei, mimicking the *in vivo* phenotype, as a result of interactions with HUVECs, which were able to organise into capillary-like structures (Salerno *et al.*, 2011).

In addition, ECs (rat brain microvascular ECs) have also been used in co-culture with neural cells (like neurons, astrocytes, and microglia) aiming at creating models of the blood-brain and the blood-spinal cord barriers as potential screening tools for pre-clinical drug discovery (Achyuta *et al.*, 2013; Watson *et al.*, 2013).

Moreover, co-culturing ECs with cardiomyocytes is also a commonly used strategy to pre-vascularise engineered cardiac tissues *in vitro*. For instance, Garzoni *et al*. developed a 3D model of tissue-like spheroids through a co-culture of cardiomyocytes and HUVECs, reporting the formation of vascular-like structures, which in turn contributed for the maintenance of long-term survival, as well as the contractile ability of the cardiac microtissue (Garzoni *et al.*, 2009). Together, these studies show that each cell type works as an integrated unit inside a complex system constituted by cells, soluble factors and matrix components.

Overview on Translational Issues and Clinical Concerns

The translation of vascular tissue engineering products to clinical use implies not only the development of a functional vascular network, but also a rapid anastomosis with the host vasculature. Hence, this topic addresses some of several issues that need to be solved for a successful translation to be achieved (L'Heureux *et al.*, 2007; Duncan and Breuer, 2009).

Cells derived from bone marrow or peripheral blood may constitute a good cell source, as they are readily available from patients by means of bone marrow aspiration or blood collection, respectively, allowing for an autologous strategy. Nonetheless, if cells are used in a heterologous way, donor to donor variation constitutes a problem that needs to be addressed in this field. For instance, graft-*versus*-host disease has been a major concern in bone marrow transplantation. For some applications, where MSCs can be applied, their immunosuppressive activity might be beneficial for patients. Although MSCs can develop into perivascular cells or even osteoblast-like cells, as seen in previous sections, further studies might be helpful in elucidating immune responses to these co-implantation systems.

On the other hand, cell therapies usually require an *in vitro* expansion step in order to obtain a sufficient number of cells for transplantation, which results in a substantial manipulation of cell products and, thus, the clinical use of cell-based therapies is subjected to their approval as Advanced Therapy Medicinal Products by certified agencies (George, 2011).

In addition, although the support provided by 3D artificial matrices is not extensively addressed in this review, the development of biomaterials that can guide the *in vivo* formation of blood vessels, assuring cell survival and attachment, is a critical step for efficiently engineering and regenerating complex 3D tissues.

Overall, these are major examples of the essential milestones that still need to be considered before the clinical application of the described strategies.

Conclusions and Future Directions

Cells are the true engineers in tissue regeneration. Cellular strategies constitute a promising way to achieve vascularisation of tissue engineering constructs avoiding the undesirable side effects of delivery of growth factors, cytokines, hormones or other bioactive molecules.

EC transplantation procedures rely on the use of support cells to assure the stabilisation of newly formed vasculature. However, the clinical use of distinct cell types would require different isolation procedures, resulting in an increased morbidity of donor sites. Vascular tissue engineering requires advanced technological strategies to be developed, as different cell types need complex interactions to orchestrate vascularisation processes in very distinct organs in the human body.

In a bench setting, co-culture systems are a useful instrument providing new insights into the molecular mechanisms underlying vascularisation. Future research is needed on the use and optimisation of these co-culture systems, which might allow not only to develop implantable vascular tissue engineered constructs, but also to understand the interplay in heterotypic cultures in order to clearly define the cues involved in host cell recruitment to the injury site, in order to improve tissue integration and regeneration.

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Discussion with Reviewers

M. Herrmann: Endothelial progenitor or blood mononuclear cells are very heterogeneous populations but obviously the interaction of these cells with mature endothelial cells is essential for blood vessel formation and repair. Is it better to use pure populations of individual cells types or can we profit from these natural interactions, what is your opinion?

Authors: We consider that the use of mixed endothelial cell populations might be beneficial in terms of their interactions to promote new blood vessels formation. Indeed, as, for instance, EPCs and OECs have been reported to influence angiogenic processes through distinct mechanisms, the use

of a mixture of these cell types could positively influence vascularisation *in vivo*, as also described by Silva *et al*. (2008; text reference). The same can happen when mature ECs are present, as they already have a functional endothelial phenotype. Indeed, the incorporation of OECs into capillary-like structures previously formed by mature ECs has also been reported (*e.g.*, Sieveking *et al*., 2008; text reference). Thus, these distinct populations seem to act/interact in a synergistic manner.