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Review

# *In vitro* models for the evaluation of angiogenic potential in bone engineering

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Blood vessels have a fundamental role both in skeletal homeostasis and in bone repair. Angiogenesis is also important for a successful bone engineering. Therefore, scaffolds should be tested for their ability to favour endothelial cell adhesion, proliferation and functions. The type of endothelial cell to use for *in vitro* assays should be carefully considered, because the properties of these cells may depend on their source. Morphological and functional relationships between endothelial cells and osteoblasts are evaluated with co-cultures, but this model should still be standardized, particularly for distinguishing the two cell types. Platelet-rich plasma and recombinant growth factors may be useful for stimulating angiogenesis.

**Keywords:** endothelial cells; bone; angiogenesis; scaffold; osteoblasts; vascular endothelial growth factors

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## Role of angiogenesis in bone engineering

In bone, the connection between cells and blood vessels is required to maintain skeletal integrity. In tissue engineering, a vessel network is an essential pre-requisite for scaffolds to survive and integrate with existing host tissue.

### Activators and inhibitors of angiogenesis

Vascular development is a co-ordinated process through three major steps, regulating (1) sprouting of endothelial cells (ECs) from mature vessels, (2) assembly of vessels to vascular structures and (3) vessel maturation and subsequent induction of quiescence[1]. Each of these steps is regulated by molecules acting on specific vascular receptors. Sprouting is induced by vascular endothelial growth factor (VEGF)[2], which is produced by monocytes and macrophages migrated to the site of the tissue lesion and stimulated by hypoxia. Vessel cells become sensitive to VEGF after the hypoxia-induced bond of angiopoietin-2 to the endothelial receptor tyrosine kinase Tie-2. VEGF binds to receptors VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR) on EC membrane. Assembly of vessels to vascular structures is regulated by the ephrin ligands and ephrin receptor tyrosine kinases, which mediate cell-contact-dependent signalling[3]. Angiopoietins[4] and Tie-1 and -2 receptors[5] regulate blood vessel maturation too. Angiogenesis is also modulated by other growth factors (GFs), cytokines, enzymes and integrins, such as fibroblast growth factor 2 (FGF-2)[6], hepatocyte growth factor/scatter factor[7], platelet derived growth factor (PDGF)[8], interluekin-8 (IL-8)[9], IL-3[10], $\alpha_v\beta_3$-integrin[11] and matrix metalloproteinases (MMPs)[12], which degrade extracellular matrix (ECM) facilitating EC migration.

### Angiogenesis and bone

In bone, microvessels are essential for bone formation, metabolism, healing and remodelling. Osteoprogenitors are always located near blood vessels. Sinusoids surrounded by reticular cells secrete high amounts of chemokine CXCL12 or stromal cell-derived factor-1 (SDF-1), which is required for the maintenance of human stem cells[13]. Both intramembraneous and endochondral bone ossification occur in close proximity to vascular ingrowth. In intramembranous ossification there is an invasion of capillaries that transport marrow stromal cells (MSCs), which differentiate into osteoblasts and in turn deposit bone matrix. In endochondral ossification the avascular cartilage template is replaced by highly vascularized bone tissue. The immature and proliferating chondrocytes secrete angiogenic inhibitors, while hypertrophic chondrocytes produce angiogenic stimulators, such as VEGF, fibroblast growth factor (FGF)-1 and FGF-2, thus providing a target for capillary invasion and angiogenesis. Hypertrophic chondrocytes and migrating cells from the newly formed bone marrow secrete

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metalloproteinases (MMPs), which in turn degrade extracellular matrix (ECM), thus permitting vessel invasion. MMP-9 regulates also the release of VEGF-A bound to the hypertrophic cartilage matrix. Once released, VEGF-A binds to its receptors on endothelial cells, osteoclasts and osteoblasts[14]. The new vasculature supplies a conduit for the recruitment of cells involved in cartilage resorption and bone deposition[15]. ECs produce GFs which contribute to recruit stem cells and to address them towards osteoblast differentiation.

Blood vessels play a crucial role in both phases of bone remodelling. In bone resorption, vessels transport osteoclast precursors to the sites of remodelling[16]. In bone deposition, vessels transport osteoprogenitor cells[17]. According to their phenotype, ECs produce molecules modulating bone remodelling, such as RANKL, osteoprotegerin (OPG), IL-6, PDGF, transforming growth factor-β (TGF-β), and others.

Angiogenesis is fundamental for fracture repair. One of the earliest events during bone healing is the reconstruction of intraosseous circulation[18]. Following trauma, disruption of vessels leads to acute hypoxia of the surrounding tissue, as well as to clotting activation. The inflammatory response activates cytokines and GFs that recruit MSCs and ECs to the fracture site. The latter produce PDGF-BB, which contributes to MSC recruitment[19]. Lack of angiogenesis is considered as a pathogenetic cause of non-unions[20].

Angiogenic GFs also play roles in bone formation. VEGF seems to play a key role in endochondral ossification[21], where its functions are mediated by cbfa-1/runx-2[22]. VEGF production is increased by BMP-2, -4, and -6[23], and by TGF-β1[24]. VEGF-A binds to VEGFR-1 on osteoclasts and induce osteoclast recruitment and bone-resorption[25]. FGF-2 stimulates the proliferation and differentiation of osteoblasts[26] and accelerates fracture repair when added to the early healing stage[27].

### Relationships between endothelial cells and osteoblasts

ECs and osteoblasts (OBs) communicate through three mechanisms[28]:

1. Direct interaction between membrane molecules of the two adjacent cells (tight junctions);

2. Gap junction communications that form direct cytoplasmic connections between adjacent cells;

3. Secretion of diffusible factors that diffuse freely in the extracellular environment and interact with the target cells through specific receptors.

Gap junction communications are mediated by Cx43 on OBs and ECs, and by Cx40 on ECs[29, 30].

Some diffusible factors secreted by ECs favour bone deposition, such as PDGF-AB, TGF-β1, TGF–β2, FGF-2, epidermal growth factor (EGF), OPG, and bone morphogenetic protein 2 (BMP-2)[31]. When ECs are incubated with a proinflammatory stimulus, such as TNF, IL-1β, or endotoxin, they synthesize both substances inducing bone healing, such as endothelin-1[32], and molecules favouring bone resorption, such as IL-6 and RANK-L[33]. Moreover, ECs may stimulate osteoblasts to express ALP[34].

In turn, MSCs produce angiogenic GFs, such as VEGF, particularly under hypoxic conditions, FGF-2, insulin-like growth factors (IGF), PDGF, and TGF-β. VEGF-stimulated ECs release prostaglandins that strongly promote VEGF release by osteoblasts[35]. ECs co-cultured with OBs show an increased production of collagen type I[36].

The interaction between ECs and OBs is variable with time, as it was shown in co-cultures onto a scaffold made of starch and polycaprolactone. At early time points ECs formed monolayer patches above OBs. At 21 days, ECs had organized into microcapillary-like structures, which were established among OBs. The concentration profile of VEGF during 35 days *in vitro* was characterized by 3 distinct phases: (1) from day 7 to 14 a steep increase in VEGF concentration; (2) between day 14 and day 28 a plateau phase and (3) from day 28 until day 35 a pronounced decrease of VEGF concentration. In hOB monoculture, the VEGF concentration curve exhibited a steady increase at lower magnitude as compared to co-culture until day 28 followed by a decrease[37].

The sonic hedgehog (Shh) pathway is involved both in bone repair and in neoangiogenesis. Hedgehog morphogens play a pivotal role in embryonic development[38]. There is increasing evidence that the Shh pathway plays a significant role in adults both in angiogenesis[39, 40] and in endochondral bone formation[41].

## Endothelial cells

ECs are classified into macrovascular and microvascular, according to the vessel type.

### Human umbilical vein endothelial cells and other macrovascular endothelial cells

HUVEC are the most known among macrovascular ECs. Other macrovascular ECs were isolated from human saphena or from human, bovine or swine aorta or pulmonary artery. However, the latter EC types have been scarcely used for the evaluation of scaffolds for bone engineering. Moreover, non-human ECs show a different behaviour than human cells[42].

### Microvascular endothelial cells

There is evidence that ECs from different organs exhibit different responses to stimulants - particularly, macrovascular ECs have different properties from microvascular ECs.

Microvascular endothelial cells (HMVEC) were isolated from adipose tissue (ADEC)[43], derma (HDMEC)[44] or lung microvessels (HPMEC)[45]. The advantages of ADEC or HDMEC consist in more similar properties to bone microvessels than HUVEC. Moreover, in light of a possibile clinical application, they may be easily isolated from the same patient who will receive the scaffold.

### Endothelial progenitor cells

EPCs are adult progenitor cells that can differentiate into mature ECs[46] and therefore play a physiological role in vessel homeostasis[47]. EPCs may be identified through the expression of three cell markers (CD133, CD34, and VEGFR-2)[48]. EPCs are mainly located in bone marrow and can be mobilized

into peripheral blood[49], where they are present from 0.01% to 0.0001% of mononuclear cells (MNCs) in healthy subjects[50]. In culture, two distinct types of EPCs develop. The first type, named early EPCs[51], appears after 3–5 days, is formed by spindle-shaped cells and dies after 4 weeks. The second type, named late EPCs[52] or outgrowth endothelial cells (OECs) [51], appears after 2–3 weeks, forms a cobblestone monolayer and lives for about 12 weeks. Early EPCs, which derive from $CD14^+$ MNCs, are myeloid cells with some endothelial properties, which stimulate neovascularization by paracrine factors but are not incorporated in the endothelial lining. OECs derive from $CD14^-$ MNCs, have similar properties to mature ECs but a higher proliferative ability[53], and are incorporated into the endothelial lining of new blood vessels[54].

One of the most therapeutically interesting features of EPCs is their apparently enhanced ability to be incorporated into newly forming microvasculature. Although their concentration in blood is low, they have been detected in newly formed vasculature, contributing about 5%–35% of the endothelial cells in new capillaries[55]. In fact, EPCs are mobilized by tissue ischemia and cytokines from the bone marrow into peripheral blood, migrate to regions of neovascularization, differentiate into mature endothelial cells and promote vasculogenesis[56]. The most known application of EPCs is the promotion of the therapeutic neovascularization in myocardial infarction[57] and liver disorders[58]. Moreover, it was shown that EPCs develop a favorable environment for fracture healing via angiogenesis and osteogenesis, through two mechanisms. One is the osteogenic and endothelial differentiation potential of CD34+ cells, and the other one is the paracrine effect of CD34+ cells, which secrete VEGF[59]. For this reason, EPCs were investigated to specifically address the problem of delayed and atrophic nonunions[60].

### Endothelial cell continuous cell lines

Typically, EC cultures are primary cultures and the proliferative potiential gradually decreases during passages. Therefore continuous cell lines were generated from angiosarcomas, or through cell immortalization with viral transfection or with fusion with neoplastic lines. Examples of tumour-derived endothelial cell lines are ISO-HAS from human haemangiosarcoma[61] and HAEND derived from hepatic angiosarcoma[62]. Transfection may be obtained with SV40 virus or with the introduction of human telomerase reverse transcriptase (hTERT)[63]. Examples of lines obtained with transfection are EVLC2, derived from HUVEC[64], HMEC-1, developed by transfection of HDMEC with SV-40 large T-antigen[65], and HPMEC-ST1.6R, developed by transfection of HPMEC with plasmids encoding the SV-40 large T-antigen and human telomerase[66].

A line obtained from the fusion of ECs with neoplastic cells is EA.hy926, which was developed from the fusion of HUVEC with human pulmonary adenocarcinoma A549[67].

The changes resulting in the capacity of cells to replicate indefinitely may be accompanied by changes in the expression of specific endothelial properties, such as the induction of inter-cellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1) or E-selectin with an inflammatory stimulus[68]. Among EA.hy926, EVLC2, HAEND, HMEC-1, ISOHAS-1, only HPMEC-ST1.6R exhibited the major constitutive and inducible endothelial cell characteristics and showed an angiogenic response on Matrigel[69].

A continuous EC line was isolated from the microvessels of bovine foetal sternus[70]. The endothelial phenotype was shown by the presence of von Willebrand factor and by the ability to form tubular-like structures on Matrigel. These cells display own distinctive characteristics, particularly they possess the receptor for estrogens and are able to respond to estrogens and parathyroid hormone (PTH)[71–73].

## Evaluation of the angiogenic potential of the scaffold

Upon graft implantation, inflammation, which represents the first phase of tissue repair, favours a vascular response, but angiogenesis is generally limited to less than 1 mm from the interface implant-host tissue[74]. Moreover, the capillary network induced by the inflammatory process is transient and regress within a few weeks[75]. Neoformed vessels of the implant must anastomose to the systemic circulation[76]. In the absence of a vascular supply, the transport of nutrients occurs mainly by diffusion, which is only efficient for distances from 100 to 200 micron or for tissues with a low metabolic activity, such as cartilage[77]. The insufficient vascularization compromises the supply of oxygen and nutrients to the new-formed tissue and does not remove the waste products of cells[78]. The local accumulation of toxic substances may trigger an inflammatory reaction[79].

Therefore the scaffold must not only support the growth of the cells that will replace the specific tissue *in vivo*, but it must also support EC adhesion and proliferation, and develop an effectively functioning vasculature to supply the cells with oxygen and nutrients. The success of this strategy requires a series of consecutive events: a) EC migration to the outer surface of the scaffold, b) EC migration from the outer surface to the inner pores of the scaffold, c) EC adhesion to the foreign surface and proliferation, d) ECM synthesis, e) tubular structures formation, f) recruitment of further cell types (smooth muscle cells, pericytes, fibroblasts) forming the vessel wall and g) anastomosis with the vessels of the surrounding tissue (Figure 1). Moreover, endothelial cells growing on the scaffold should maintain normal functions and should not exhibit a pro-inflammatory phenotype. In order to obtain stable and durable vascular networks, ECs require the cooperation with perivascular cells. It was shown that a network of stable and functional blood vessels was formed in mice by co-implantation of vascular ECs and mesenchymal precursor cells[80].

In the light of the critical role of angiogenesis, a preliminary step in the evaluation of the scaffold properties should predict its vascularization potential through the assessment of its interaction with ECs.

### Endothelial cell seeding

ECs are seeded on the scaffold, which may be pre-condition-

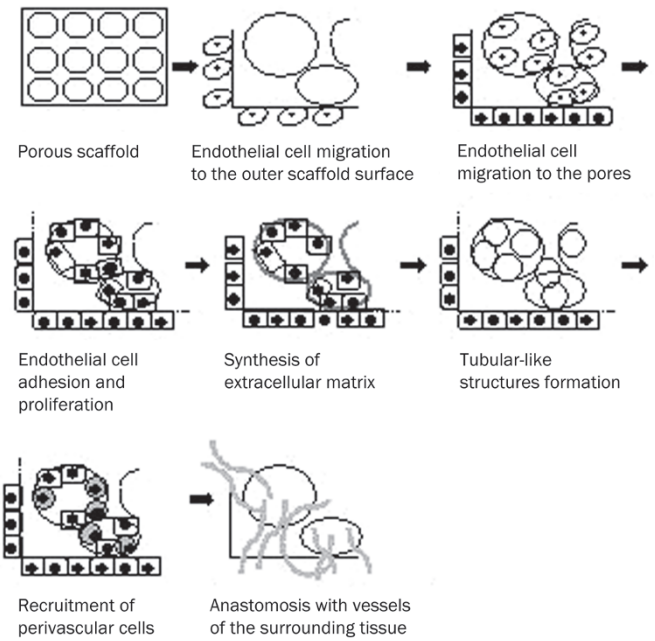


**Figure 1.** Neoangiogenesis on a porous scaffold.

ated by immersion in culture medium for a few hours. This treatment leads to protein adsorption on the artificial surface, and purposes to reproduce the ECM layer to which the cells usually adhere to live on. Cell seeding is favoured by the following technique: to apply a drop of cell suspension on the scaffold, to incubate at 37 °C for 15–30 min in a wet chamber to allow initial cell attachment, then to add culture medium. The scaffold may be coated with gelatin or fibronectin or collagen or pooled serum to favour cell adhesion.

### Endothelial cell morphology

Cell morphology can be evaluated before or after staining. Immunofluorescence for specific antigens, such as von Willebrand factor or CD31, or stain with fluorescent molecules, such as calcein-acetoxymethylester[81] or acridine orange, may be used. Cytoskeleton is shown by F-actin stain (Figure 2A)

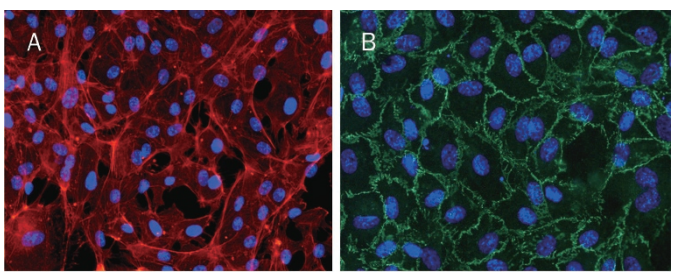


**Figure 2.** Immunofluorescent staining of endothelial cells for proteins specific for cytoskeleton or for adhesion molecules. (A) F-actin stain of HUVEC. Actin was stained using rhodamine-phalloidin fluorescent dye. Nuclei were stained with Hoechst 33258. Magnification (×20). (B) Immunofluorescent staining for VE-cadherin. Nuclei were stained with Hoechst 33258. Magnification (×20).

or by immunofluorescence for vimentin[82]. VE-cadherin is an adhesion molecule that mediates cell-to-cell contact between endothelial cells and plays a relevant role in the maintenance of vascular integrity[83] (Figure 2B). The maintenance of VE-cadherin expression on a scaffold is a good indicator of the proper interaction between endothelial cells and the material. Fluorescent staining of intracellular organelles may be evaluated by confocal microscopy. With fluorescence microscopy and scanning electron microscopy the relationships between cells and scaffold may be investigated, but no information is obtained on the biomaterial effects on the synthesis of specific molecules.

### Adhesion and spreading

The scaffold could affect the synthesis of molecules for homotypic adhesion (CD31, VE-cadherin) or of integrin for the adhesion to substratum[84]. To favour EC and osteoblast adhesion, RGD-peptides were bound to the scaffold. These peptides are formed by the arginine, glutammic acid and aspartic acid sequence, which is common to the integrin $\alpha_v\beta_3$ ligands[85]. Moreover, the scaffold could decrease the expression of adhesion molecules for leukocytes (E-selectin, ICAM and VCAM) in response to inflammatory stimula or, *vice versa*, could induce their expression also on basal conditions[86]. Cell adhesion to the substratum and adhesion molecules are evaluated at fluorescence and confocal microscopy with immunofluorescence staining[87].

A quantitative method to evaluate the expression of adhesion molecules is an enzyme immunoassay performed directly on the cells adherent to the substratum (CAM-EIA)[88]. Flow cytometry is another quantitative assay for adhesion molecules and integrins, but it requires cell detachment, which could affect their expression[89]. Moreover, adhesion molecules and integrins may be indirectly evaluated with RT-PCR for their specific mRNA[90].

### Cell proliferation

The proliferation of endothelial cells on artificial scaffolds may be directly determined with vital stains, such as blue alamar[91] or calcein-acetoxymethylester[92], without detaching cells. Alamar changes from blue to pink in proportion to the amount of reactions of oxide-reduction of the cells and therefore in proportion to the cell number. Through a standard curve with known cell numbers, the cell number of the unknown sample grown on the scaffold can be obtained with a good approximation[93]. Calcein-acetoxymethylester becomes fluorescent when taken up by viable cells and the fluorescence is spread throughout the cell. Alternatively the cell number can be evaluated with tritiated thymidine[94], MTT[95], or crystal violet[96] assays.

### Tubulogenesis

EC grown on the scaffold can be evaluated for their ability to form tubular-like structures. ECs are seeded on an inducing matrix, with or without GFs. This matrix can be used also to coat the scaffold. After a few hours, tubular-like structure

formation is observed, eventually after vital staining with calcein-acetoxymethylester and nuclear staining with Hoechst 33342[97]. The tube number, length and bifurcations may be quantificated with image analysis, in order to appreciate differences among scaffolds. Matrigel (Becton Dickinson), formed by EMC proteins, is a well-known matrix. Also type I collagen was used, which determined after 20 h the formation of tubular-like structures similar to capillaries[97].

### Expression of proteins acting on bone remodelling

For the evaluation of scaffolds for bone engineering, the assay of GFs, cytokines and other proteins produced by endothelium and acting on bone remodelling could be useful. Both specific mRNA expression[98] and the concentration of these substances in the conditioned medium are determined[99]. Proinflammatory markers should be assayed both on basal conditions and after incubation with LPS, because the biomaterial could affect the cell response to the proinflammatory stimulus[100].

## Co-cultures

The different co-culture systems take the different interactions among ECs and OBs into account (Figure 3):

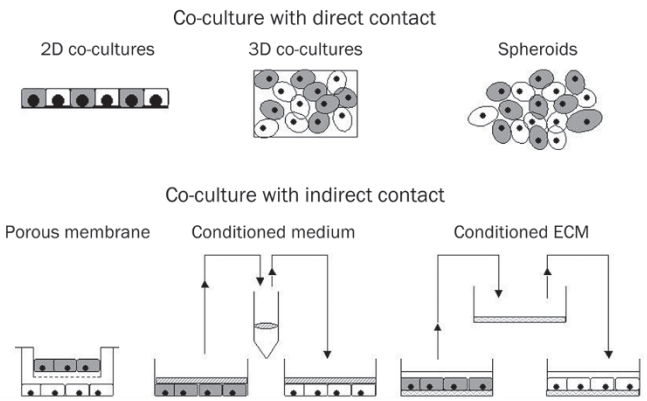


**Figure 3.** Cell co-culture systems. In the direct contact model, cells are seeded together in 2D supports or 3D scaffolds or as 3-D multicellular spheroids. In the indirect contact model, a porous membrane can be used, with appropriate pore size which let the conditioned medium pass but not cells. Alternatively, the culture of one type of cells is supplemented with the conditioned medium of the other type. In the third method, one cell type is seeded on the ECM of the other type, which has been discharged after grown.

- Co-cultures with direct contact can be initiated on a 2-D surface or on a 3D scaffold or with spheroid systems. With direct contact all the mechanisms of cell-to-cell interaction are evaluated (direct interaction through tight junctions, gap junction communications and secretion of paracrine factors)[28];

- Co-cultures with indirect contact evaluate only communications through paracrine soluble factors. The two cell types may be put in indirect contact through a porous membrane with a such pore size which lets the conditioned medium pass but not cells[101]. Other methods consist in culturing one cell type in the conditioned medium of the other type or in seeding a cell type over the ECM that has been produced by the other cell type.

The indirect contact methods allow to easily quantificate the reciprocal metabolic influences, but do not give any information on the reciprocal spatial relationships.

With the direct contact method the relationships between cells into the living tissue are simulated. 2D studies provide detailed information of the molecular basis of cell-to-cell contacts, and knowledge of cellular events governing the differentiation of OBs that are in contact with ECs. Conversely, 3D co-cultures offer a physiologically optimized environment for cell survival which favors the formation of functional blood vessels. Spheroids formed when a cellular suspension in medium containing 20% methyl cellulose (Methocel, Dow Chemical Co, USA) was seeded in nonadhesive wells with U shape[102]. HUVECs were grown as 3-D multicellular spheroids in a collagen matrix. Direct cell contact between hOBs and HUVECs was established by incorporating hOBs into the EC spheroids, thus forming heterogeneous cospheroids. Co-culture spheroids differentiated spontaneously to organize into a core of OBs and a surface layer of ECs[103].

Direct contact method requires the standardization of a) the choice of culture medium, b) the choice to seed the cell types at the same time or successively, and which type should be seeded as the first; c) the ratio between cell types, d) the separation of the cell types during and at the end of the co-cultures. As culture medium, the medium of ECs, which have higher nutrition requirements, is usually chosen. Both cell types may be seeded at the same time or ECs are seeded before OBs. The optimal ratio between HDMVEC and OBs was shown to be 5:1 or 10:1[92]. The evaluation of the relationships between the cell types is based on differential staining with quantum dots incorporated by cells before seeding and the evaluation at fluorescence or confocal microscopy. Also staining for von Willebrand factor or CD31, specific for ECs, and ALP, specific for OBs, were used. Time-lapse microscopy showed the formation of a tubular-like network through the movement of HUVEC along hOB and their philopodia[104].

With morphological methods the relationships between cells are investigated, but no information on the reciprocal metabolic influences is obtained. The latter is examined with the assay of specific genes and protein of each cell type. ECs produce VEGFR-1, VEGFR-2, CD31, Tie-1, Tie-2, but do not synthesize collagen I, ALP and osteocalcin. ECs co-cultured with hOB stimulated ALP activity and mineralization[105], but down-regulated runx2, osteocalcin and Cx43[106]. The most serious problem is the evaluation of the relative synthesis of proteins because the total protein content of each cell type cannot be distinguished. Also gene expression cannot be normalized because housekeeping genes are common to hECs and hOB, unless human and animal cells are co-cultured. Therefore it is fundamental to separate the two cell types at the end of co-culture. Magnetic immunoseparation with anti-CD31 antibodies showed upregulation of 79 genes and downregula-

tion of 62 genes in OBs and, particularly, the downregulation of the gene of *PDGF receptor* α after co-culture with ECs[101].

Direct co-culture of ECs and OBs prevented the precoating of biomaterials with gelatin or fibronectin. Moreover, ECs formed an extensive network of capillary-like structures with lumina only when they were co-cultured with OBs, but not when they were cultured on biomaterials alone even in the presence of an exogenously supplied angiogenic stimulus. Thus, a prevascularization can take place *in vitro* only in the presence of OBs[92].

## Local delivery of angiogenic growth factors

Biomaterial vascularization can be promoted or by seeding mature ECs or their progenitors directly on scaffolds[107], or by locally delivering angiogenic GFs[108], which may induce ECs to migrate, proliferate, and produce molecules acting on bone remodelling. Particularly, it was shown that VEGF-A increased mRNA specific for FGF-2 and decreased mRNA specific for IL-6[19], and increased both mRNA expression and surface protein expression of RANK[109].

### Delivery of recombinant growth factors

The ability of biodegradable scaffolds to locally deliver GFs mimicks the conditions of tissue repair *in vivo*. A combination of PDGF-BB and VEGF-165 initiated formation and maturation of a significant number of blood vessels[110]. When calvarial defects of rats were treated with scaffolds in poly(*D*,*L*-lactide-co-glycolide) (PLGA) bound to rhVEGF-A, a significant increase of blood vessel formation, bone coverage, and bone mineral density was observed in comparison with defects treated with simple PLGA[111]. The use of rhVEGF-A in bone defect models showed that new blood vessel formation preceded the osteogenic front and that an increased angiogenesis corresponded to an increased bone formation[112]. However, the local application of VEGF-A to rabbit tibia during distraction osteogenesis increased the blood flow in the distracted limb, but failed to influence bone mineral content and histomorphometric indices of bone regeneration[113]. A possible explanation was that a high level of endogenous VEGF-A had already been secreted during osteogenesis, reaching an optimal local concentration, and therefore the additional delivery of VEGF-A had little or no effect[114].

### Platelet-rich plasma (PRP)

A more feasible way to administer angiogenic GFs consists in the application of PRP. In fact, activated platelets release osteogenic and angiogenic GFs from α-granules, such as PDGF, TGF-β, IGF, EGF, and VEGF. Therefore autologous platelets activated with thrombin were used as a source of GFs to stimulate tissue repair. PRP could also favour proliferation and differentiation of the cells seeded on scaffolds, ECs included[115], also when they were co-cultured with hOB[116].

### Gene therapy

*Ex vivo* gene therapy, which consists in the transplantation of genetically modified MSCs secreting angiogenic GFs[117], could overcome the limits of conventional GF delivery. When adipose-derived stem cells (ADSCs) were transfected with adenovirus encoding the cDNA of VEGF, the combination of VEGF releasing cells and ECs resulted in a higher vascular growth within PLGA scaffolds[118].

## Discussion

In the last forty years, the new knowledge in cellular and molecular biology and the possibility of the synthesis of innovative materials have determined a shift from the concept of an "inert" material, *ie* non-toxic for cells and tissues of the body, to the concept of a "bioactive" material, which favours cellular adhesion, proliferation and functions. At the same time, it has been understood that angiogenesis was necessary not only for the treatment of obstructive vasculopathies, but also for the repair of most tissues and organs. Consequently, it has been understood that the successful clinical outcome of an implanted cell-construct is dependent on the establishment of a functional vascular network. Therefore, scaffolds should be tested for their angiogenic potential before implantation. Particularly, the ability to favour EC adhesion, proliferation and functions should be assayed with *in vitro* and *in vivo* tests.

The choice of the EC type is crucial, because they may have different properties according to their source. For the evaluation of scaffolds intended for bone engineering, cells with similar characteristics to ECs of bone vasculature should be chosen. Olfactory ensheathing cells (OECs), which can be isolated from the patients without invasive methods, seem to be the most suitable in the formation of functional vessels anastomosed to the host's vascular system[119].

Among the different models of *in vitro* evaluation of the angiogenic potential, the co-cultures between ECs and OBs are the closest to the *in vivo* situation. However, they allow to appreciate the reciprocal relationships between these cell types, but still need to be standardized for the quantitative evaluation of specific gene expression and protein synthesis.

At present, *in vitro* models alone cannot predict if the capillary-like structures pre-formed in the scaffold will establish connections *in vivo* with the host microvascular system. A co-culture of EPCs and MSCs on a scaffold followed by implantation in animal demonstrated improved osteogenesis and angiogenesis when the scaffold had been seeded with the two cell types, without ischemic necrosis at the center of the graft, while impaired osteogenesis and progressive necrosis were observed when the scaffold had been seeded with only OBs[120].

At present, efforts are mainly focused on stabilizing neovasculature and thus promoting the formation of lasting blood vessels. Perivascular cells such as pericytes and smooth muscle cells contribute to the remodelling and maturation of the primitive vascular network and therefore are fundamental agents in the construction of a durable engineered vasculature. In this line of thought, the actual co-culture systems will be upgraded with tri-cultures among OBs, ECs and perivascular cells.

In conclusion, blood vessels, which are necessary to skel-

etal homeostasis and bone repair, have a fundamental role to assure the incorporation of a cells-scaffold construct into the body. Therefore, the ability of the scaffolds to favour EC adhesion and proliferation, without affecting their functions, should be assayed. In *in vitro* tests, the source of ECs should be carefully considered, because it may affect their properties. Morphological and functional relationships between ECs and OBs should be evaluated with appropriate models, such as co-cultures. PRP and recombinant GFs may be useful for stimulating neoangiogenesis.

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## Author contribution

Elisabetta CENNI wrote the manuscript; Francesca PERUT contributed to the sections Endothelial progenitor cells, Evaluation of the angiogenic potential of the scaffold, and Local delivery of angiogenic growth factors, and to the photographs at fluorescence microscopy; Nicola BALDINI supervised the paper.

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