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# Improving vascularization of engineered bone through the generation of pro-angiogenic effects in co-culture systems☆

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ABSTRACT

One of the major problems with bone tissue engineering is the development of a rapid vascularization after implantation to supply the growing osteoblast cells with the nutrients to grow and survive as well as to remove waste products. It has been demonstrated that capillary-like structures produced in vitro will anastomose rapidly after implantation and become functioning blood vessels. For this reason, in recent years many studies have examined a variety of human osteoblast and endothelial cell co-culture systems in order to distribute osteoblasts on all parts of the bone scaffold and at the same time provide conditions for the endothelial cells to migrate to form a network of capillary-like structures throughout the osteoblast-colonized scaffold. The movement and proliferation of endothelial cells to form capillary-like structures is known as angiogenesis and is dependent on a variety of pro-angiogenic factors. This review summarizes human 2- and 3-D co-culture models to date, the types and origins of cells used in the co-cultures and the proangiogenic factors that have been identified in the co-culture models.



**Contents**



## 1. Introduction

Generating healthy bone for the replacement of diseased or missing bone tissue has been one of the major objectives of tissue engineering scientists in the last years. The bone consists of a mineralized organic matrix containing living cells, which together form the rigid component of the vertebrate skeleton. In addition, similar to most other tissues and organs, the bone is interlaced with blood vessels through which a

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continuous flow of blood supplies the cells making up the tissue or organ with the essential nutrients and oxygen to survive, while at the same time removing $CO_2$ and waste products. Moreover, in the case of bone, the vasculature also delivers the calcium and phosphate needed for the mineralization process. Thus, to generate new bone, a suitable matrix needs to support the growth and phenotype-specific functioning of the cells making up the bone, such as osteoblasts, osteocytes and osteoclasts as well as the endothelial cells integral to the blood vessels.

Tissue engineering is a therapeutic approach in regenerative medicine which combines a "bio" material, cells and other factors to generate a tissue-like structure. For bone, much initial research has been dedicated to finding and optimizing biomaterials which allow the attachment, growth and differentiation of osteoblasts. In particular, the goal of this research has been to identify the material composition which shows the best mineralization on the biomaterial by the added osteoblast cells [1–3]. However, the success and survival of a biomaterial after implantation is also dependent on the outcome of the vacularization process. Not only do the bone cells need to grow, spread and differentiate on the biomaterial, but a rapid neo-vascularization and blood flow to and within the implant must take place. Initially, after implantation, delivery of nutrients to cells on a biomaterial is limited to approximately 200 μm and occurs via interstitial fluid diffusion [4–8]. Obviously most bone tissue regeneration applications are considerably larger, this being the case after tumor surgery. Thus a slow or incomplete vascularization would result in an inadequate oxygen and nutrient supply leading to hypoxia and cell death of cells implanted with the material or preventing the ingrowth of bone cells from the host [9,10]. This has been confirmed in studies showing a substantial loss of osteoblasts due to insufficient initial vascularization within the first week following implantation of a porous cancellous bone biomaterial [11].

It is evident from the above that if the tissue-engineered construct does not contain or allow a rapid ingrowth of blood vessels, the implanted biomaterial will not survive. Overcoming this hurdle has been one of the major areas of focus in regenerative bone tissue engineering strategies. In recent years, a substantial amount of research has been dedicated to generating a network of capillary-like structures on a biomaterial in vitro. It has been shown that pre-formed capillaries in vitro have a structure containing a lumen and tight junctions similar to capillaries in vivo [12–15]. Furthermore, these capillaries can anastomose with the host vasculature after implantation and supply blood to the biomaterial construct [16–19].

## 2. Improving vascularization of engineered bone in vitro

### *2.1. Formation of the vasculature: vasculogenesis and angiogenesis in vivo and in vitro*

A vascularized tissue is one through which blood flows via a network of capillaries. These capillaries are primarily made up of endothelial cells. Blood microcapillaries are formed in vivo by two major mechanisms, vasculogenesis and angiogenesis. Vasculogenesis is the formation of new capillary-like structures from individual cells not associated with pre-existing capillaries [20,21]. Angiogenesis, on the other hand, is the formation of new capillaries via sprouting from pre-existing capillaries [5]. In vitro, "vasculogenesis" occurs when individual endothelial cells dispersed on a surface or in a 3-D scaffold migrate and come together to form microcapilliary-like structures containing a lumen. With time an interconnected network of capillaries may form [20,21]. Over prolonged incubation times sprouting from the newly-formed microcapillaries may occur, indicative of angiogenesis. Sprouting of monolayers of endothelial cells on flat surfaces or imbedded in 3-D gel-based spheroids or microbeads mimics angiogenesis as observed in vivo [22].

In both vasculogenesis and angiogenesis, specific proangiogenic factors are required for endothelial cells to migrate and form microcapillaries. These microcapillaries cannot be generated by endothelial cells themselves. Therefore, single cell cultures of endothelial cells require exogenously added proangiogenic compounds to form microcapillary-like structures [23,24]. Many compounds within different molecular families have been identified that stimulate and influence angiogenesis such as vascular endothelial cell growth factor (VEGF), basic fibroblast growth factor (bFGF), vascular endothelial cell growth factor receptors (VEGFR), angiopoeitin 1 and 2 (Ang-1 Ang-2), platelet-derived growth factor (PDGF), transforming growth factor-beta (TFG-β), endoglin, transforming growth factor-beta receptors (TGF-β receptors), various integrins, VE-Cadherin and platelet endothelial cell adhesion molecule (PECAM-1, CD31) in addition to many others. These compounds can stimulate proliferation, differentiation, capillary stability, recruitment of other cell types and matrix reorganization (for reviews see [5,20–22,25,26]). In co-cultures, the cell–cell interactions (or cellular crosstalk) of bone and endothelial cells results in the production of the required proangiogenic factors. The following reviews the study of cell co-culture systems containing human osteoblasts and endothelial cells isolated from a variety of tissue sources that have been developed with the goal of generating a bone-like biomaterial for implantation. The focus of this review is threefold: (1) describing the models resulting in a pre-formed microcapillary network in vitro, (2) identifying proangiogenic factors that could play a role in the formation of these structures, and (3) determining whether implanted materials exhibit an anastomosis with the host vasculature, a process called inosculation.

### *2.2. Bone and endothelial cells for co-culture systems for bone regeneration*

#### *2.2.1. Sources of human endothelial cells*

A variety of endothelial cell types have been isolated from the body. The most widely used are the easily obtainable HUVECs (human umbilical vein endothelial cells) followed by HDMECs (human dermal microvascular endothelial cells, generally isolated from human foreskin tissue) [27,28]. Primary macro- and microvascular endothelial cells have been isolated from a large number of other tissues such as adipose, aortic, lung, and brain tissues [29–32]. Macro- and microvascular endothelial cells from different sources share many common phenotypes, although clear differences in gene expression patterns and structures do exist, depending on location or type of endothelial cell [33–35]. A special example is given by endothelial cells making up the blood–brain barrier, as they exhibit exceptionally tight junctions and transport mechanisms not seen at other locations in the body [36]. Although primary cells can generally be isolated and cultivated relatively easily they are not the optimal choice for tissue regenerative purposes due mostly to their limited lifespan. In recent years, endothelial-like cells known as endothelial progenitor cells (EPCs) have been isolated from blood and these are a promising source of endothelial cells for tissue engineering purposes [37,38]. These cells are isolated from the mononuclear fraction of cells derived from peripheral blood buffy coats using antibodies against specific markers on the cell surface of cells isolated, such as CD31+, CD34+ or CD133+, or by selection of adherent cells arising after culture in endothelial cell-specific differentiation media. The endothelial cells isolated by these methods are commonly known as early and late outgrowth endothelial cells (OECs) [12]. Although early and late outgrowth endothelial cells share similarities there are differences in the proliferation rates, with only late outgrowth endothelial cells having the proven ability to form microcapillary-like structures [39].

Mesenchymal stem cells (MSCs) can be differentiated to chondrocytes, adipocytes or osteoblasts, although recent studies have demonstrated that MSCs isolated from the bone marrow or blood, as well as other sources, can also differentiate and yield cells expressing an endothelial cell-like phenotype under specific culture conditions [40–42]. Endothelial cells have also been generated from embryonic stem (ES) cells and show endothelial cell morphology, gene expression and angiogenic