

## ENGINEERING THICK TISSUES – THE VASCULARISATION PROBLEM

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### **Abstract**

The ability to create thick tissues is a major tissue engineering challenge, requiring the development of a suitable vascular supply. Current trends are seeing the utilization of cells seeded into hybrid matrix/scaffold systems to create *in vitro* vascular analogues. Approaches that aim to create vasculature *in vitro* include the use of biological extracellular matrices such as collagen hydrogels, porous biodegradable polymeric scaffolds with macro- and micro-lumens and micro-channels, co-culture of cells, incorporation of growth factors, culture in dynamic bioreactor environments, and combinations of these. Of particular interest are those approaches that aim to create bioengineered tissues *in vitro* that can be readily connected to the host's vasculature following implantation in order to maintain cell viability.

**Key Words:** co-culture, hydrogel, extracellular matrix, scaffold, lumens, channels, bioreactors, vascularisation, tissue engineering.

### **Introduction**

#### **The Thick Tissue Dilemma**

As defined by the National Science Foundation, "Tissue engineering is an interdisciplinary field that applies the principles of engineering & the life sciences towards the development of biological substitutes that restore, maintain or improve tissue function." (Skalak and Fox, 1988). A critical obstacle in tissue engineering is the inability to maintain large masses of living cells upon transfer from the *in vitro* culture conditions into the host *in vivo*. *In vivo* most cells do not survive more than a few hundred micrometers from the nearest capillary, due to diffusion limitations (Frerich *et al.*, 2001; Okano and Matsuda, 1998; Sheridan *et al.*, 2000). Capillaries, and the vascular system, are required to supply essential nutrients, including oxygen, remove waste products and provide a biochemical communication "highway" (Cassell *et al.*, 2002; Eiselt *et al.*, 1998; Nerem *et al.*, 1998; Nguyen and D'Amore, 2001; Nomi *et al.*, 2002; Secomb and Pries, 2002). The circulatory system also contributes to the control of temperature and pressure/perfusion through vasoconstriction and vasodilation. The cardiovascular system is also the earliest to develop in the embryo (Auerbach and Auerbach, 2002; Nguyen and D'Amore, 2001) and the establishment and maintenance of a vascular supply is an absolute requirement for the growth of both normal and neoplastic tissues (Auerbach and Auerbach, 2002; Gridley, 2007). All tissues and organs (with the exception of a minority of tissues such as cartilage) need to be vascularised to be able to survive. Generating a vascular supply to a thick tissue-engineered construct remains a major challenge (Griffith *et al.*, 2005; Grikscheit and Vacanti, 2002): new blood vessels take days to develop (Griffith and Naughton, 2002) which can lead, for example, to predisposed ischaemia (Grounds, 2000). This review will examine the process of vascularisation *in vivo* and then explore the current methods of vascularising bioengineered tissues and organs *in vitro*, which include the use of microvascular cells and co-culture, and biological and synthetic scaffolds, and explore a novel approach to creating a vascularised tissue analogue *in vitro*.

### **The Vascular System**

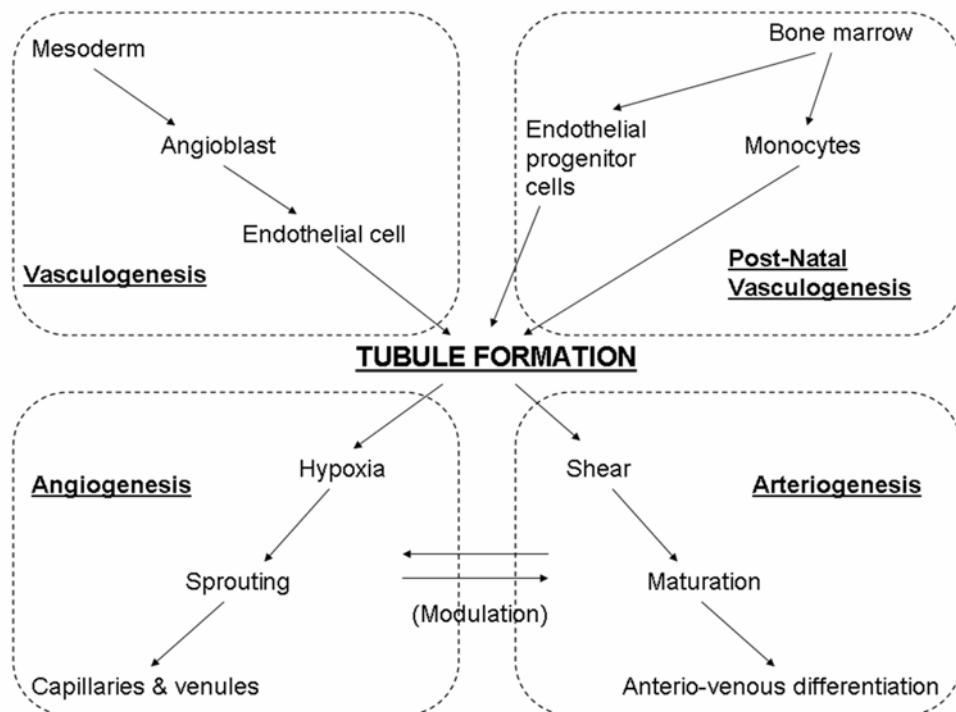
#### **Vascularisation**

Blood vessels are constructed by two processes, angiogenesis, which is a morphogenic process involving the sprouting of capillaries from pre-existing blood vessels, and vasculogenesis, which is the *in situ* assembly of capillaries from undifferentiated endothelial cells (EC). Adult blood vessel formation may be a combination of

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**Figure 1.** The relationship between the various pathways to vessel development. Adapted from (Kannan, *et al.*, 2005).

the two (Cassell *et al.*, 2002; Nguyen and D’Amore, 2001), with the main target vessels for angiogenic stimuli being the post-capillary venules and the small terminal venules.

The angiogenic process requires close interactions between the extracellular matrix (ECM), cells and growth factors to produce the required neovascularisation (Cassell *et al.*, 2002). There are six basic steps in angiogenesis: vasodilation, basement membrane degradation, adherent cell migration, formation of capillary lumen, synthesis of basement membrane, and recruitment of pericytes and vascular smooth muscle cells (Ahrendt *et al.*, 1998; Auerbach and Auerbach, 2002; Cassell *et al.*, 2002; Downs, 2003; Egginton and Gerritsen, 2003; Folkman and Haudenschild, 1980; Jain *et al.*, 1997; Nomi *et al.*, 2002; Norrby, 1997; Vailhe *et al.*, 2001).

The vasculogenesis process, like the angiogenesis process, can be divided into six steps (Auerbach and Auerbach, 2002; Nomi *et al.*, 2002; Vailhe *et al.*, 2001):

1. The *in situ* differentiation of mesodermal cells into angioblasts or hemangioblasts.
2. EC are generated from precursor cells (angioblasts).
3. EC form the vessel primordia and aggregates that establish cell-to-cell contact but have no lumen.
4. A nascent endothelial tube is formed, composed of polarized EC.
5. A primary vascular network is formed from an array of nascent endothelial tubes.
6. Pericytes and vascular smooth muscle cells are recruited.

Figure 1 illustrates the key processes involved in tubule formation and vascularisation of tissues in animals (Kannan *et al.*, 2005).

Once vessels are made, remodelling of the vascular network occurs in response to the physiological demands

of the system. Remodelling of the vascular system involves changes in lumen diameter and wall thickness to suit local needs (Cassell *et al.*, 2002) and can occur through differentiation of the microvascular wall. Differentiation of the microvascular wall can be related to (1) mechanical factors, primarily the blood hydrostatic pressure that acts on arterioles, and (2) metabolic factors related to the local metabolic needs of tissues and reflected in peculiarities of blood-tissue exchange vessels (capillaries and small venules) (Slaaf *et al.*, 1993). Intussusceptive microvascular growth (IMG) is a concept of vascular morphogenesis and remodelling that provides an alternative mechanism to angiogenesis by endothelial sprouting. IMG refers to partitioning of the perfused vessel lumen by the insertion of interstitial (or intervacular) tissue structures or tissue pillars. This is followed by growth of these structures resulting in network expansion. IMG is also an important mechanism of vascular network remodelling, defined as rearrangement of the number and/or position of vascular segments without significant network expansion (Jain *et al.*, 1997; Patan *et al.*, 2001). IMG, however, has not been observed so far in vascular analogue tissue engineering research. So in order to identify an *in vitro* bioengineered vascular analogue as functional, the created vasculature has to be able to respond to both mechanical and metabolic factors.

#### Blood vessel structure

The smallest microvessels are the capillaries with an inner diameter of 4–10 µm and a very thin wall of EC. The wall thickness of microvessels can be as much as half the luminal diameter. The boundary between arteriole and capillary is marked by the disappearance of smooth muscle cells (SMCs). The arterioles represent the major site of

resistance to and regulation of local blood flow, with arterioles varying in diameter from 10 $\mu\text{m}$  to 300 $\mu\text{m}$ , with one or two layers of SMC helically arranged in the media, with the inner lining consisting of endothelium (Slaaf *et al.*, 1993). However the transition from capillary to venule is less well defined and occurs gradually. The immediate post-capillary venules range in diameter from ~10-50 $\mu\text{m}$  and initially lack SMC in their walls. They drain into collecting venules, the diameter of which gradually increases from ~50-300 $\mu\text{m}$  (Slaaf *et al.*, 1993).

Large vessels are separate anatomical entities, whereas microvessels are structurally and functionally part of the tissue they supply for which the input for the microcirculation of tissues is small arteries (Slaaf *et al.*, 1993). Immature endothelial-lined tubes that arise during vasculogenesis and angiogenesis subsequently differentiate into capillaries (after association with pericytes) or into larger vessels such as arteries and veins (after forming a media composed essentially of SMC).

### Generation of vascular structures

Traditionally, research on vascularisation has relied on animal models to observe and understand the process (Norrbry, 2006). Various models exist such as the chorioallantoic membrane model (Borges *et al.*, 2003b) and the disc angiogenesis system (Kowalski *et al.*, 1992), as well as the analysis of the expression of various factors associated with vascularisation after implantation of scaffolds into animals such as mice (Vallien *et al.*, 2000).

There has been some success in creating vascular analogue scaffolds and precursor tissues *in vivo*. Implanted cell-seeded constructs in animals have acted as living bioreactors for cells within scaffolds, and models of vascularisation in animals (Cassell *et al.*, 2002; Koike *et al.*, 2004) have included adapted chorioallantoic membrane (Borges *et al.*, 2003a), porcine (Schultheiss *et al.*, 2005), mice (including nude mice) (Koike *et al.*, 2004; Okano and Matsuda, 1998; Sieminski *et al.*, 2002), and immunodeficient mouse (Nor *et al.*, 2001) models.

Ischemia has also been shown to stimulate vascularization. Circulating endothelial progenitor cells (EPCs) have been mobilized endogenously in response to tissue ischemia or exogenously by cytokine therapy and seem to augment neovascularization of ischemic tissues (Takahashi *et al.*, 1999). Circulating mononuclear cells in human peripheral blood have been shown to be incorporated into foci of neovascularization, consistent with postnatal vasculogenesis. In experiments, the development of regional ischemia in both mice and rabbits increases the frequency of circulating EPCs (Takahashi *et al.*, 1999).

### Vascular Analogues *In Vitro*

#### Creation of vascular networks and mass transfer considerations

The question of whether vascularisation can occur *in vitro* without a blood supply is an important one. From a functionality viewpoint, if we define a bioengineered vessel

as being viable when it can mimic everything a natural vessel does *in vivo*, then we are currently disappointingly short of that ideal target. Currently in biological and synthetic scaffolds, *in vitro* formation of multi-branching networks of cells (Kaihara *et al.*, 2000; Wu *et al.*, 2004), lumens (Egginton and Gerritsen, 2003; Sieminski and Gooch, 2004; Sieminski *et al.*, 2004), contiguous vessel walls (Black *et al.*, 1998), and responsiveness to chemical and biophysical signals (Lee *et al.*, 2000), have been observed. But nothing so far has been able to compare to the effectiveness and quality of microvascular network formation *in vivo* (Borges *et al.*, 2003a; Patan *et al.*, 2001). This may be due in part to the complex combination of biochemical signals in blood and the biophysical influences that accompany blood flow (Lee and Schmid-Schonbein, 1995; Pries and Secomb, 2002). Unless we are able to elucidate and simulate the complex biochemical cocktail in blood, as well as the biophysical environment during blood flow, we may well need a blood supply to create a functional microvascular system.

Pre-capillary and vessel networks can occur in various forms *in vitro* and on various substrates and materials (Kannan *et al.*, 2005; Moldovan and Ferrari, 2002; Vailhe *et al.*, 2001). Two regulatory pathways have been proposed by which angiogenesis is thought to proceed. The proliferative pathway depends on various cytokines and other factors that both stimulate and inhibit the proliferation of EC. The morphogenetic pathway depends on the synthesis and assembly of fibrillar type 1 collagen, which can be used as a template for EC migration and lumen formation. EC interact with substrates of type 1 collagen and form networks based on the establishment of traction centres. These planar cellular networks, in some respects, resemble developing vasculature *in vivo* (Sage and Vernon, 1994).

The use of microvascular EC (MVEC), the key cells for vascularisation in the body, for angiogenesis *in vitro* was demonstrated in 1980 (Folkman and Haudenschild, 1980) and subsequently by others (Bachetti and Morbidelli, 2000). Some groups have been able to reconstruct semi-vascularised bioengineered tissues and equivalents *in vitro*, including “vascularised” skin (Black *et al.*, 1998; Germain *et al.*, 2000; Tonello *et al.*, 2003), while other groups have created cell models for vascularisation and capillary-network formation to study various cell-cell, cell-matrix (Sieminski *et al.*, 2004) and cell-nutrient diffusion interactions (Griffith *et al.*, 2005).

There has been much work done on creating functional macrovessels using bioreactors that utilize perfusion and mechanical stimulation (Barron *et al.*, 2003; Jeong *et al.*, 2005; McCulloch *et al.*, 2004; Mironov *et al.*, 2003; Niklason *et al.*, 2001; Niklason *et al.*, 1999; Stegemann and Nerem, 2003; Thompson *et al.*, 2002). There is no doubt that bioreactors are essential for the *in vitro* creation of functional and viable tissues and vessels (Freed and Vunjak-Novakovic, 2002; Martin *et al.*, 2004; Niklason and Seruya, 2002). For microvascular applications, some groups have created microvessels using perfusion bioreactor technologies (Neumann *et al.*, 2003), while others have used horizontal rotating bioreactors, such as the one developed by NASA (Dutt *et al.*, 2003).

A traditional research area for investigating the functionality of microvessels has been the use of the eye system, for example in the diabetic retinopathy field. The modulation of the transport barrier of MVEC (Haselton *et al.*, 1996) is definitely one of the defining functions of the microvasculature. Functional responses to ischemia could be simulated in culture environments to influence arteriogenesis, collateral vessel formation, and increase blood vessel density once bioengineered tissues are vascularised (Helisch and Schaper, 2003).

However, comprehensive vascularisation of thick tissues *in vitro* is hard to control and reproduce reliably. From this point there is the next limitation of creating proper connections of the bioengineered tissue with host vasculature when the tissue is implanted.

### Cell Types for *In Vitro* Vascular Analogues

#### Microvascular and macrovascular endothelial cells

In tissue engineering vasculature, stabilization of capillary-like structures (CLS) is of utmost importance (Frerich *et al.*, 2001). Endothelium is most important in the microcirculation, where exchange of gases, fluids, nutrients, and waste products occurs. The endothelia of capillaries most commonly exist as a continuous sheet, but also can take the form of a sheet with fenestrations (in small intestines, endocrine glands, and kidneys), or as a discontinuous sheet (in sinusoids of the liver) (Nguyen and D'Amore, 2001; Sumpio *et al.*, 2002).

Permeability regulation is important for microvessels and capillaries (Haselton *et al.*, 1996; Hellstrom *et al.*, 2001; Nguyen *et al.*, 2001; Sumpio *et al.*, 2002). Fenestrations, caveolae, and pores are at the level of the EC soma as well as gaps between EC. Fenestrations are specialised areas of the EC plasma membrane that are involved in permeability, and fenestrated endothelia are found in microvessels associated with secretory and absorptive epithelia, such as kidney glomeruli, endocrine organs, and the gastrointestinal tract (Nguyen *et al.*, 2001; Sumpio *et al.*, 2002). In contrast, EC found in connective tissues, skin, muscle, lung, and brain have few, if any fenestrations (Nguyen *et al.*, 2001).

Endothelial cells exert significant paracrine and endocrine actions through their influence on the underlying SMC or on circulating blood elements, such as platelets and white blood cells (Sumpio *et al.*, 2002). EC are normally quiescent *in vivo* and most in adults have a cell cycle variable from months to years. However, following an injury, cells change their phenotype, migrate and proliferate to heal lesions in a few days (Bachetti and Morbidelli, 2000).

EC also play direct roles in controlling the vascular tone, blood flow and vascular permeability, especially in the microvasculature (Bachetti and Morbidelli, 2000). Various types of EC such as human umbilical vein EC (HUVEC) (Tonello *et al.*, 2003), rat microvascular EC (Frye and Patrick, 2002), human dermal microvascular EC (HDMEC) (Borges *et al.*, 2003a; Nor *et al.*, 2001; Richard *et al.*, 1998), bovine aortic EC (BAEC) (Dutt *et al.*, 2003;

Vernon *et al.*, 1995) and bovine capillary EC (BCEC) have been used for *in vitro* models of angiogenesis and vasculogenesis (Karasek, 1989; Vailhe *et al.*, 2001). The immortalised HMEC line SV-40 HMEC-1 has also been used (Ades *et al.*, 1992; Liu *et al.*, 2002; Meade-Tollin and Van Noorden, 2000). Gelatin, fibrin, collagen, fibronectin (Harding *et al.*, 2002) and Matrigel (Meade-Tollin and Van Noorden, 2000) have been used as matrices for 2D and 3D culture studies. It has been noted though that cultured EC rapidly undergo apoptosis following implantation *in vivo* (Nomi *et al.*, 2002).

The biochemical profile of microvascular and macrovascular EC differ, indicating system-specific functions and roles. For example the matrix metalloproteinases (MMP) and tissue inhibitor of metalloproteinases (TIMP) secretory profiles of cultured microvascular compared to macrovascular tissues is different (Jackson and Nguyen, 1997). For example cytokeratin 8, 18, 19 and vimentin are cytoskeletal proteins that are present on microvascular EC but not on macrovascular EC (Bachetti and Morbidelli, 2000). The ECM components produced by microvascular EC and macrovascular EC are different, and are linked to the different thrombogenic properties of the cells (Bonnefoy *et al.*, 2001). On a wider application focus, there are differences in phenotype expression between primary cells and cell lines as well (Unger *et al.*, 2002).

#### Interaction of endothelial cells with smooth muscle cells

Even though SMC are not typically associated with capillaries, endothelial progenitor cells (EPC) have been co-cultured with SMC to form microvessels on porous polymer scaffolds *in vitro* (Wu *et al.*, 2004) and it has been reported that rat aortic SMC can become pericyte-like during angiogenesis *in vitro* (Nicosia and Villaschi, 1995). It has also been reported that, like EC, vascular SMC can display blood vessel formation behavior *in vitro* when an appropriate 3D matrix environment is provided to keep them in a relatively higher-differentiated and low-proliferative state (Song *et al.*, 2000). An artificial method to induce migration, and potentially CLS formation, of EC has been to co-culture EC with VEGF-transfected SMC or their conditioned media (Elbeirami and West, 2006). The significance of these observations for tissue engineering is that SMC may be used in bioengineering microvascular networks. However, the importance and biological relevance of SMC over pericytes remains to be determined since pericytes are the key supporting cells in microvascular networks *in vivo*.

#### Pericytes

The origin of cells may be an important factor when utilizing cells for specific tissues, and it is important to use cells with the correct phenotype (Jackson and Nguyen, 1997). For undifferentiated cells though, this may not be the case. For example, pericytes have been suggested to have tissue-specific functions (Hirschi and D'Amore, 1996). Although tissue-specific cell functions can be shown from cells that display plasticity, as has been demonstrated

by various “tissue-specific” cell types (Rezai *et al.*, 2004; Tao and Ma, 2003).

Pericytes, also known as Rouget cells or mural cells, are the microvascular correlates of SMC, lie within the basement membrane and have processes that reach through to contact the EC (Hirschi and D’Amore, 1996; Thomas, 1999). Morphologically, pericytes exhibit a small, oval cell body with multiple processes extending for some distance along the vessel axis. These primary processes then give rise to orthogonal secondary branches which encircle the vascular wall. Pericytes are precisely located adjacent to or over EC junctions of venules and especially over gaps between EC during inflammation (Sims, 2000). Of functional importance, it has been reported that pericyte inclusion in *in vitro* capillary permeability experiments increases the capillary barrier effect (Dente *et al.*, 2001).

Perivascular cells (pericytes and SMC) stabilise newly formed capillaries and render them to matured structures (Hirschi and D’Amore, 1996). Pericytes are most abundant in venules, and common in capillaries (Nguyen *et al.*, 2001; Sims, 2000), and are suggested to be oligopotential and reported to differentiate into adipocytes, osteoblasts, macrophages, and phagocytes (Hirschi and D’Amore, 1996; Thomas, 1999). Microvessel pericytes seem to exhibit several oxygen dependent, phenotypic characteristics ascribed to osteoblasts (Reilly *et al.*, 1998). However, they are absent from growing vessels, implying a role in down-regulating EC proliferation (Cassell *et al.*, 2002). Vessels that are associated with pericytes persist independently of VEGF, whereas those lacking pericytes regress following VEGF withdrawal (Carmeliet and Conway, 2001; Carmeliet and Luttun, 2001).

The pericyte population is highly variable between different tissues and organs, likely influenced by post-arteriolar hydrostatic pressures, and are more abundant in the distal legs and feet, suggesting a hydrostatic pressure-driven mechanical role for pericytes as protectors of microvessel wall integrity (Sims, 2000). This implies a major role for perivascular mesenchymal cells for the stabilization of newly formed vascular structures, and that the use of perivascular cells is likely to be beneficial for *in vitro* vascular bed systems, and to help create and maintain a functional *in vitro* vascular bed template. Pericytes may also function as macrophages, amongst other functional roles. They can be contractile and seem to serve as a smooth muscle equivalent in the capillaries performing vasoconstriction, and they appear to directly participate in coagulation through the extrinsic pathway (Thomas, 1999).

It has also been seen *in vivo* that monocytes and macrophages can have the propensity for endothelial trans-differentiation within Matrigel and may have a role in formation of CLS (Anghelina *et al.*, 2006a; Anghelina *et al.*, 2006b).

### **Stem cells & bone marrow stromal cells (BMSC)**

Bone marrow-derived cells have been reported to induce repair of tissue *in vivo* (Zantop *et al.*, 2006; Zhou *et al.*, 2007). For vascularisation and re-vascularisation purposes, precursor cells, such as endothelial precursor cells, have

been used to study angiogenesis (Laing *et al.*, 2007a; Laing *et al.*, 2007b; Otani *et al.*, 2002). BMSC have been used for therapeutic angiogenesis in animal research for many years (Choong *et al.*, 2006; Kaigler *et al.*, 2006a; Kruyt *et al.*, 2007; Short *et al.*, 2003; Zhou *et al.*, 2007). These can form blood vessel-like structures consisting of endothelial tubes supported by SMC in 3D matrix gels and in chick embryo models (Nomi *et al.*, 2002), retinal precursor cells in ocular angiogenesis experiments (Dutt *et al.*, 2003), and EPC for *in vivo* ocular disease models (Otani *et al.*, 2002).

Adult bone marrow (BM) contains cells capable of differentiating along hematopoietic (Lin(+)) or non-hematopoietic (Lin(-)) lineages. Lin(-) hematopoietic stem cells (HSCs) have been shown to contain a population of EPC capable of forming blood vessels (Otani *et al.*, 2002). In neonatal mouse eye experiments, Lin(-) BM cells and astrocytes specifically interact with one another during normal angiogenesis and pathological vascular degeneration in the retina, and are extensively and stably incorporated into the forming retinal vasculature (Otani *et al.*, 2002).

Current data suggest stromal progenitors in the BM *in situ* are associated with the outer surfaces of blood vessels and may share identity with vascular pericytes (Short *et al.*, 2003). In adult organisms, BM-derived cells do not promote vascular growth by incorporating into vessel walls but may function as supporting cells (Ziegelhoeffer *et al.*, 2004). The major contribution of BM cells to angiogenic processes is not endothelial, but may come from progenitors for periendothelial vascular mural and hematopoietic effector cells (Rajantie *et al.*, 2004).

Autologous BMSC implantation in a chronic hind limb ischemia model in rats seems to induce a neo-vascular response resulting in a significant increase in blood flow to the ischemic limb, as well as spontaneously regenerating the various components of muscular tissues (Al-Khalidi *et al.*, 2003). Use of BMSC and EC in scaffolds has seen the regeneration of osseous defects (Kaigler *et al.*, 2006a). The relevance to vasculogenesis of these observations is that use of BMSC would have benefits for both vascularisation and whole tissue and organ regeneration because BMSC could be used for vascularisation and organ tissue growth. EPC as well as other BM-derived cells have been shown to contribute to the growth of endothelium-lined vessels (angiogenesis) as well as the expansion of pre-existing collaterals (arteriogenesis) in ischemic disease (Carmeliet and Luttun, 2001). In another study, the microvessel density in ischemic hindlimbs in mice was significantly higher in CD117<sup>+</sup> cell-implanted mice than in the total BM cell-implanted mice, and implicated as playing a key role in therapeutic angiogenesis induced by BM cell implantation (Li *et al.*, 2003).

Other BM-derived cells such as BM-derived fibroblasts and BM endothelial cells have been used in 3D scaffold cultures with the aim of stimulating vascularisation and tissue formation (Choong *et al.*, 2006; Srouji *et al.*, 2006).

### **Co-culture**

The interaction of EC with other cell types *in vivo* has been seen to promote and maintain vascularisation. The

aim of using co-culture is to mimic the beneficial and complex interactions between cells to create and maintain vascularisation *in vitro*. However, the optimal combination of cells, their ratios, and conditions are still unknowns.

When blood vessels grow, EC migrate out first and assemble in a primitive network of immature channels (angiogenesis). These nascent vessels only consist of EC, and they rupture easily and are leaky, prone to regression, and poorly perfused. If perfusion is to become functional, the network must remodel into a vascular tree of mature large vessels branching off into smaller capillaries, whereby excess immature vessels are pruned. The recruitment of periendothelial cells (PEC) (pericytes in small vessels or SMC in larger vessels) around nascent vessels essentially contributes to the remodelling and maturation of the primitive vascular network (arteriogenesis) (Dente *et al.*, 2001; Hirschi and D'Amore, 1996; Sims, 2000). When PEC make contact with EC, the EC stop dividing and migrating, and EC become quiescent, acquire specialised differentiation properties, and survive for longer (Carmeliet and Conway, 2001; Sims, 2000). PEC stabilize vessels by producing ECM and tightening junctions, regulating perfusion through vasomotion, establish vascular barriers, and make vessels more resistant to regression (Thomas, 1999), and they also help to establish vascular branches and prevent aneurysmal vessel dilation. PEC help to establish a more mature, functional vascular network and are therefore targets for inducing therapeutic angiogenesis (Carmeliet and Conway, 2001).

In co-culture studies of BAEC and human astrocytes, regulation of extracellular angio-associated migratory cell protein (AAMP) by astrocytes has been hypothesized to aid in angiogenesis of the nervous system (Beckner *et al.*, 2002).

Microvascular EC can also be used for other purposes. Porcine brain microvascular EC have been used to support the *in vitro* expansion of human primitive hematopoietic BM progenitor cells via cell-to-cell interactions and colony-stimulating factors (Davis *et al.*, 1995). These examples highlight the complex roles that microvascular cells play in vascular biology. It is therefore foreseen for *in vitro* applications that the interactions between different cell types in co-culture may provide beneficial cues for forming stable vessels and tissues.

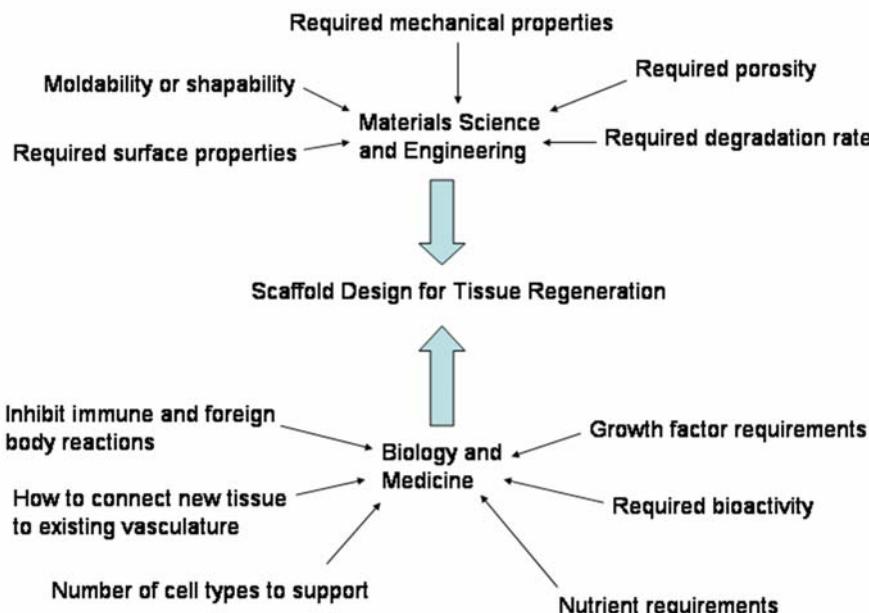
Additional questions for co-culture utilization for vascularisation *in vitro* are not simply questions about cell-cell interactions or cell-matrix interactions, but in situations involving synthetic scaffolds, are the interactions and behaviours of co-cultures with the scaffold material surface (Choong *et al.*, 2006). Fibroblasts are another cell type that has been used in co-culture. Fibroblasts not only function to sustain various organs and tissues as stroma cells but also act directly to regulate adjacent cell behavior including migration, proliferation, and differentiation. *In vitro* results in collagen hydrogels suggest that fibroblasts secrete soluble factors that can influence EC behaviours relevant to the angiogenesis process with possible implications for vascularization in fibroproliferative conditions (Kuzuya and Kinsella, 1994). Without the necessary survival signals, EC suspended in 3D gels *in*

*vitro* die, but the presence of non-EC improves, for example, the survival of BAEC embedded within Matrigel (Sieminski *et al.*, 2002). EC have been shown to elongate and form microvascular networks along lines of ECM tension in fibroblast-containing culture gels (Sieminski *et al.*, 2002), and culture of BAEC with fibroblasts causes them to elongate and enhance their survival *in vitro* compared to control cultures without any other cells. A mechanism for improved cell survival has been hypothesized to be the direct physical contact between BAEC and the fibroblasts (Kuzuya and Kinsella, 1994; Sieminski *et al.*, 2002). In a further example, in a disc angiogenesis model with EC and fibroblasts, angiogenesis is observed (Kowalski *et al.*, 1992), and the results support the need for co-culture for effective cell functioning.

Dermal fibroblasts are reported to produce angiogenic growth factors (Griffith and Naughton, 2002). BAEC co-cultured in collagen hydrogels with human skin fibroblasts cause the EC to become spindle shaped and to organize into a CLS within the collagen hydrogels. Interestingly, fibroblast-conditioned medium also induces EC initially to elongate and subsequently to organize into a CLS within collagen hydrogels (Kuzuya and Kinsella, 1994). In endothelialized skin equivalents, collagen biopolymer co-cultured with human keratinocytes, dermal fibroblasts, and umbilical vein EC have spontaneously formed CLS in a highly differentiated ECM (Black *et al.*, 1998). *In vivo*, culture of human dermal microvascular EC (HDMVEC) spheroids and preadipocytes in a fibrin matrix for a specially adapted chorioallantoic membrane model have resulted in vascularisation of the matrix, with a capillary network forming consisting of the transplanted HDMVEC (Borges *et al.*, 2003b). However, some other tissues have not experienced the same success, such as creating muscle tissue with an *in vivo* pre-vascularised fibrin matrix (Bach *et al.*, 2006). For potential neuro-regeneration applications, neurons and glioma cells have been seen to over-express stem-cell factor which induces angiogenesis *in vitro* and *in vivo* in response to tumour-induced brain injury (Thomas, 2006). Scaffold-free multi-cell artificially-created microtissues using combinations of myoblasts, chondrocytes, myofibroblasts, cardiomyocytes and HUVEC have been implanted *in vivo* and have developed a vascular system (Kelm *et al.*, 2006a; Kelm *et al.*, 2006b). This approach further illustrates the viability of utilizing complex multiple cell types in the pursuit of creating viable tissue engineered tissues.

### Approaches to vascularising scaffolds

Vascularisation of new bioengineered tissue can be accomplished in three ways. Upon implantation, vessels from surrounding tissue can be induced to infiltrate the tissue with such vessel growth promoted by including growth factors in the polymer scaffold. These factors diffuse into the local environment, where they encourage existing blood vessels to grow into the scaffold with cells growing in from both sides knotting together to form a continuous vessel (Kannan *et al.*, 2005; Sheridan *et al.*, 2000). But this approach is problematic as blood vessels need time to infiltrate into tissues, and thereby this will



**Figure 2.** Some of the biological and engineering design requirements of scaffolds for tissue regeneration. Adapted from (Seal, *et al.*, 2001).

predispose the tissue to ischemia as the cells wait for a nutrient supply to reach them. Vessels may also grow from within a scaffold seeded with EC to help enhance the rate of vascularisation. This approach is based on the hypothesis that the transplanted cells will form CLS and join with inwards growing vessels from the host tissue and combine with existing blood vessels to create a continuous vessel (Kannan *et al.*, 2005; Sheridan *et al.*, 2000). This approach is also associated with a time delay before vascular perfusion can occur due to the time for the vessel network within the scaffold to anastomose with the host vasculature. There is also a third method where matrices are pre-vascularised *in vivo* (Lokmic *et al.*, 2007; Polykandriotis *et al.*, 2006; Polykandriotis *et al.*, 2007), and though this review focuses on *in vitro* methods it is important to apply *in vivo* results and observations to *in vitro* applications.

### Use of three-dimensional scaffolds

With the possible exception of blood, three-dimensional (3D) scaffolds are essential for creating realistic tissues and organs for regenerative medicine. Tissues exist as 3D structures, but the traditional approach of growing cells in tissue culture flasks is far removed from the *in vivo* situation. Cells *in vitro* proliferate at a liquid/substratum interface and primary tissue cells grown in tissue culture flasks experience contact inhibition. This severely limits the degree of total cellularity of the system (Long, 2000).

Design factors for control of 3D tissue engineering constructs include (1) the required substrate and ECM composition (materials), (2) composition and rates of tissue development (rates), (3) cell types present, localisation and segregation from each other (cell types), (4) tissue shape and microscopic architecture (architecture), and (5) special local tissue functional requirements (Brown, 2000; Chen *et al.*, 2001a). Some of the other elements to consider are illustrated in Figure 2 (Seal *et al.*, 2001).

### Synthetic polymer scaffolds

Synthetic biodegradable polymer scaffolds have been a material of choice to create scaffolds to support cell growth for bioengineered tissues and organs because they are more controllable from a compositional and materials processing viewpoint than biological materials. The drawback is that they may not be recognised by cells due to the absence of biological signals. Scaffold architecture parameters such as pore size, porosity, and surface area (surface-to-volume ratio) are widely recognised as important parameters when designing a scaffold (Cao *et al.*, 2006; Mastrogiacomo *et al.*, 2006). Other structural features such as pore shape, pore wall morphology, and interconnectivity between pores are also important for cell seeding, migration, growth, mass transport, gene expression, and new tissue formation in 3D (Ma and Choi, 2001; Mastrogiacomo *et al.*, 2006; Mikos and Temenoff, 2000).

To coarsely mimic vasculature, some scaffold designs have incorporated grooves, microchannels and/or orientated pores to aid in directing cell growth and behaviour (Britland *et al.*, 1996; Curtis and Wilkinson, 2001; Kaihara *et al.*, 2000; Shen *et al.*, 2006a; Shen *et al.*, 2006b). This strategy also extends to biological scaffolds with lumens/channels (Sachlos *et al.*, 2006; Sachlos *et al.*, 2003). Microchannels in scaffolds and cell culture environments have been used to increase mass transfer of nutrients and cellular metabolic wastes in and out of the cell/scaffold mass (Kaihara *et al.*, 2000; Leclerc *et al.*, 2004c; Sachlos *et al.*, 2003; Shin *et al.*, 2004), and mathematical modelling has been used to create networks with specific microfluidics and nutritional transfer (Leclerc *et al.*, 2004a; Leclerc *et al.*, 2004c; Radisic *et al.*, 2006). Channels and microchannel networks not only have applications in vascularising scaffolds and bioengineered tissues (Grikscheit and Vacanti, 2002; Leclerc *et al.*, 2004c), or simply as blood vessel templates, but are also suited for neural applications in growing peripheral nerves

(Sundback *et al.*, 2003). An application has been to create branching structures of the vascular network following a fractal geometry where it has been observed that EC densities and degree of organisation are higher on scaffolds with higher branching levels, or complexity (Vozzi *et al.*, 2004).

Soft lithography techniques such as stamps or channels have been used to create scaffolds. Also used are techniques such as self-assembled monolayers, microcontact printing, patterning using microfluidic channels (Kim *et al.*, 1998b), laser microfabrication (Duncan *et al.*, 2002) and laminar flow patterning (Kane *et al.*, 1999; Leclerc *et al.*, 2004b; Moldovan and Ferrari, 2002). Benefits of these approaches are the immediate perfusion of living tissue via the integrated “vascular” network, and that these can be used to potentially create “vascularized” layered 3D structures of any shape and size (Kaihara *et al.*, 2000; Vacanti *et al.*, 2003).

Biodegradable and bioresorbable polymers have been based primarily on clinically established products, such as polylactides, polyglycolides, optically active and racemic polylactides, and polycaprolactone (Hutmacher, 2001; Hutmacher *et al.*, 2001). But there are other synthetic materials being used including ones that are under clinical investigation for regulatory approval as well as more novel polymeric biomaterials that are being tested *in vitro* only, such as peptide-based materials (Holmes, 2002). Disadvantages of cellular solid fabrication methods, using these synthetic polymers, such as solvent leaching, porogen leaching, gas foaming, vacuum drying, and thermally induced phase separation (TIPS) (Chen *et al.*, 2002; Hutmacher, 2001) are that pores are sometimes not completely inter-connected. Pores may vary in size and the thickness and length of pore walls and edges vary depending on the solvent evaporation rate. The scaffolds cannot be made with thick sections as deeply embedded porogens become too distant from the surface and residual porogen may be left in the final structure (Hutmacher, 2001). However, with the development of better techniques to ensure porogen connectivity and therefore pore interconnectivity, such as porogen fusion, there has been progress made into creating better cellular solids scaffolds (Murphy *et al.*, 2002). Alternatively, precise computer-controlled manufacturing methods such as fused deposition modelling can create porous scaffolds (Zein *et al.*, 2002). This can be used, for example, to create knitted textile scaffolds with specific woven geometries (Wintermantel *et al.*, 1996).

### **Biological scaffolds**

Biological materials are utilised in tissue engineering due to the fact that there are many beneficial interactions between these materials with cells. Natural materials are readily recognised by cells, but the drawbacks to using these materials include their poor mechanical properties. Interactions between cells and biological ECM are catalysts to cell migration, proliferation, differentiation, and apoptosis, which are all critical functions in tissues (Grikscheit and Vacanti, 2002). Mechanical strength of tissues derives in a major part from the ECM and

production of matrix, and matrix production can be manipulated (Edelman, 1999). Biomechanical tension between EC and matrix may serve to regulate capillary development since mechanical forces exerted by the cells onto the matrix drive its reorganisation into cords and subsequent CLS formation (Vailhe *et al.*, 2001).

Collagen is an important ECM molecule (Kleinman *et al.*, 1982), and is the major structural component in the body, comprising up to 25% of the dry protein (Brown, 2000). Collagen is an important part of the structure and formation of vasculature, so it makes biomimetic sense to use collagen for bioengineered vascularized tissues. Of interest for *in vitro* utilization of collagens, types I and IV are equally active both in native and denatured forms (Kleinman *et al.*, 1982). There are also very few interspecies differences in the structure of type I collagen. The extensive homology of the structure of type I collagen means that when sourced from animal species it is acceptable as a material for human transplantation (Li, 2003). For tissue engineering applications, collagen hydrogels are formed via fibrillogenesis of soluble collagen and have a random orientation and distribution, producing an isotropic gel (Brown, 2000). Porous collagen matrices are made by freeze-drying aqueous collagen solutions or dispersions. Freeze-dried matrices require chemical cross-linking to stabilize the structure where the rate of freezing, the size of fibres in the dispersion, and the presence and absence of other macromolecules contribute to the pore structure (Li, 2003).

Growth factor delivery using collagen hydrogels is another benefit of utilizing this biological material. For example, loading of basic fibroblast growth factor into heparin sulphate-attached (Pieper *et al.*, 2002) or glycosaminoglycan (GAG)-attached (Geutjes *et al.*, 2006) cross-linked collagen matrices has seen improved long term vascularisation of hydrogels *in vivo*. In mice implantation studies, VEGF incorporation into cross-linked collagen hydrogels has seen significant angiogenesis around implants (Tabata *et al.*, 2000) and into the scaffolds (Nillesen *et al.*, 2007). Cell delivery using collagen hydrogel has been utilized with, for example, a skeletal muscle cell line and type 1 collagen hydrogel scaffold constructed *in vitro* and implanted into nude mice to vascularize it *in vivo* (Okano and Matsuda, 1998) as well as with bone-marrow derived mesenchymal stem cells (BM-MSC) implanted into mice that has shown increased cellularity and vascularisation (Markowicz *et al.*, 2006). In contrast, viable tissues have also been seen in transplanted un-prevascularised cells in collagen matrices, where EC have rapidly infiltrated the tissue (Zhao *et al.*, 2005).

The relative magnitudes of cellular force generation and apparent collagen hydrogel matrix stiffness seem to modulate capillary morphogenesis *in vitro*. This balance may play a role in regulating angiogenesis *in vivo*. In constrained collagen hydrogels EC have appeared spread, formed structures with fewer cells, had larger, thinner-walled lumens than in floating (unconstrained) hydrogels, and showed prominent actin stress fibers not seen in floating gels (Sieminski *et al.*, 2004).

Collagen hydrogels have also been used extensively for engineering macrovessels for cardiovascular applications (Nerem *et al.*, 1998; Seliktar and Nerem, 2002). Approaches have included the use of cross-linked hydrogels to create mechanically strong vessels (Berglund *et al.*, 2003), use of SMC cultured in collagen hydrogels (Song *et al.*, 2000; Stegemann and Nerem, 2003a), ECs co-cultured with SMC (Imberti *et al.*, 2002), the use of mechanical stimulation regimes, such as cyclical radial distension and/or shear stress, in bioreactors (Braddon *et al.*, 2002; Chappell *et al.*, 1998; Hoerstrup *et al.*, 2001; Imberti *et al.*, 2002; Nerem *et al.*, 1998; Thomas *et al.*, 2002; Ziegler *et al.*, 1995), as well as the use of various biochemical growth factors to help create a biomimetic vessel substitute (Imberti *et al.*, 2002; Stegemann and Nerem, 2003b; Stegemann and Nerem, 2003a). There have also been studies where collagen and fibrin hydrogels loaded with VEGF and exposed to low interstitial fluid flow result in the formation of different CLS structures (Helm *et al.*, 2007) giving rise to the importance of the specific matrix composition on CLS structure.

To influence the structure of collagen hydrogels, cells in collagen hydrogels can be exposed to mechanical uniaxial stimulation to aid in aligning cells and fibres in a certain direction. High density magnetic fields have also been used to align collagen hydrogel fibres during fibrillogenesis (Dickinson *et al.*, 1994; Guido and Tranquillo, 1993), however, controlling the structure of the collagen hydrogel fibrils at such an early stage has not been conclusively beneficial. This may be because cells, in response to external cues, will respond accordingly to remodel their environment.

### Hybrid scaffolds

The need for hybrid natural/synthetic scaffolds stems from the need to meet the demands for a scaffold system that cells respond to favourably as well as being reliably and repeatedly manufacturable to a consistent quality, and have controllable structural and chemical composition for creating bioengineered organs *in vitro*. These criteria encompass elements such as:

1. a surface that promotes cell adhesion, promotes cell growth and allows the retention of differentiated cell phenotypes,
2. biocompatible, and the by-products should not be toxic or provoke inflammation *in vivo*,
3. biodegradable, and eventually eliminated from the body,
4. porosity should be high enough to provide sufficient space for cell adhesion, ECM regeneration and allow maximal diffusion during culture,
5. pore structures that allow even spatial cell distribution throughout the scaffold to facilitate homogeneous tissue formation, and
6. the material should be reproducibly processable into 3D structures, and be mechanically strong (Chen *et al.*, 2002).

The motivation for hybrid natural/synthetic scaffolds has been to combine the benefits of ease of processing,

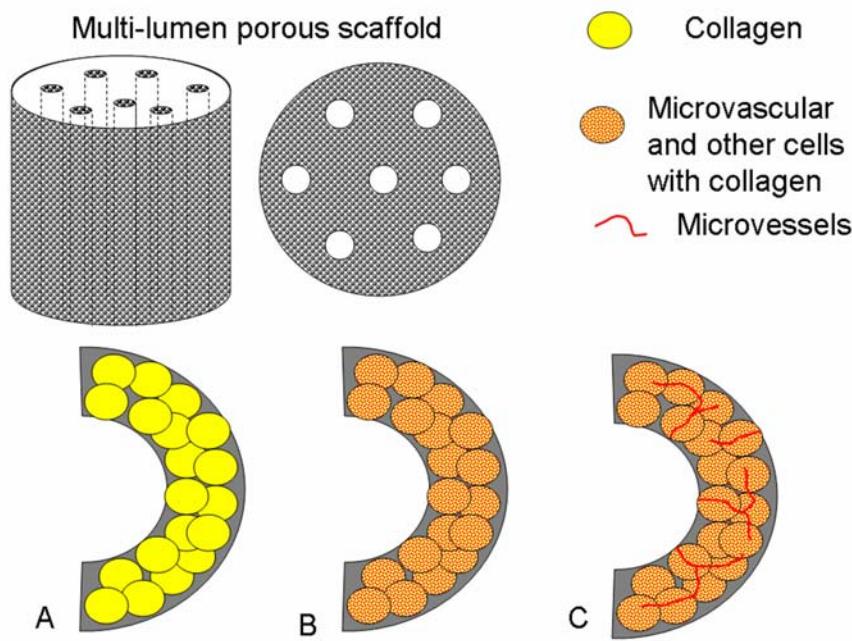
structure control, and relatively good mechanical strength of synthetic polymers, with the benefits of beneficial cell interactions and hydrophilicity of biological matrices like collagen. The most common method of hybridizing collagen with synthetic polymer scaffolds has been to coat the scaffold with a collagen gel that becomes a sponge (Chen *et al.*, 2004a; Chen *et al.*, 2003; Chen *et al.*, 2004b; Chen *et al.*, 2000; Chen *et al.*, 2001a; Chen *et al.*, 2002). Biological molecules, such as fibronectin (Harding *et al.*, 2002) and VEGF (Elcin and Elcin, 2006; Kaigler *et al.*, 2006b), have also been used to coat synthetic polymer scaffolds for growing vessels.

### Bridging the *in vivo / in vitro* gap

#### Concept

For a bioengineered organ to be clinically relevant, it needs to be able to be seamlessly integrated when implanted into the patient. There needs to be a system that prevents cell death due to the changes in perfusion quality during the transferral from an *in vitro* system (i.e. bioreactor) to the *in vivo* system (i.e. circulatory system). Currently there are techniques that vascularise pre-shaped tissues *in vivo* before autologously transplanting to another site on the body (MacLeod *et al.*, 2003; Tan *et al.*, 2004), but this causes donor site morbidity and so is therefore less than ideal.

To address these issues, we have integrated five elements from current approaches, which have been commonly used separately in the literature, as a novel approach to vascularising tissue analogues *in vitro*. The first two components are the incorporation of multiple macro-lumens lumens (Kaihara *et al.*, 2000; Leclerc *et al.*, 2004b; Sachlos *et al.*, 2006; Sachlos *et al.*, 2003; Shin *et al.*, 2004), in porogen-fused, highly porous and interconnected biodegradable synthetic polymer scaffolds (Gao *et al.*, 2006; Murphy *et al.*, 2002) to aid in increasing access to nutrients for cells within the scaffold, as well as creating “vessels” that can be used to connect to host vasculature on implantation. The third component is the use of collagen hydrogel as a cell delivery vehicle into the porous scaffolds. The collagen is also the biological matrix that will support cell growth, migration, and vascularisation behaviour of microvascular cells. Figure 3 illustrates the components of this system, where a porous polymer scaffold is combined with cells and biological matrix to direct cell behaviour and eventual tissue growth. The fourth component is the use of co-cultures of EC with other cell types to investigate the interaction between the various cells, scaffold, and formed microvessels in the system (Beckner *et al.*, 2002; Black *et al.*, 1998; Dente *et al.*, 2001; Imegwu *et al.*, 2001; Kuzuya and Kinsella, 1994). An analogue that can be created with co-culture is bone, via the incorporation of BMSC (Choong *et al.*, 2006; Kaigler *et al.*, 2006a; Kruyt *et al.*, 2007; Short *et al.*, 2003; Zhou *et al.*, 2007). In fact the use of BMSC has been reported to secrete sufficient quantities of VEGF to enhance survival and differentiation of EC *in vitro* (Kaigler *et al.*, 2003). The use of BMSC itself has been used for neovascularisation (Kinnaird *et al.*, 2004; Li *et al.*, 2003).



**Figure 3.** Components of the hybrid scaffold system based on collagen hydrogel cell delivery into a highly interconnected and porous synthetic biodegradable polymer scaffold. (A), (B), and (C) show cross-sections of the lumens and pores. (A) illustrates collagen infused into the pores of the scaffold, while (B) shows a mixture of microvascular and other cells with collagen in the pores. (C) shows the formation of microvessels in the scaffold pores from a mixture of microvascular cells with collagen hydrogel.

The fifth component is the use of a dynamic culture environment to provide biomechanical stimulation to the cells within the scaffold, as well as to provide increased mixing of cell culture nutrient media for compositional homogeneity. It has been reported that modalities such as flow perfusion (van den Dolder *et al.*, 2003) on BM cells has beneficial effects on proliferation and differentiation. The combination of high scaffold porosity and fluid flow minimizes diffusion constraints enhances differentiation of BMSC (Gomes *et al.*, 2006).

#### Collagen infusion and cell attachment

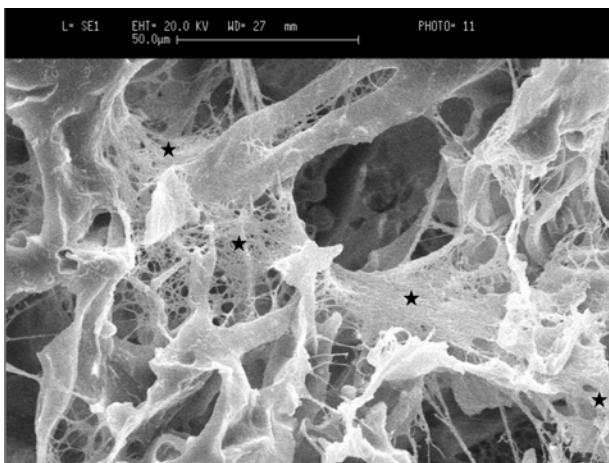
An important aspect of growing tissues *in vitro* has been for cells to be distributed homogeneously throughout the scaffold. This is a traditional consideration in animal cell culture to prevent heterogeneous localised cell growth and behaviour within the same growth environment. Methods such as dynamic perfusion (Li *et al.*, 2001; Sodian *et al.*, 2002; Wendt *et al.*, 2003), centrifugal force (Godbey *et al.*, 2004; Yang *et al.*, 2001), rotation (Nasseri *et al.*, 2003), magnetic cell seeding (Perea *et al.*, 2006) and stirring/agitation (Kim *et al.*, 1998a) have been used with some success. By using a collagen hydrogel cell delivery system, cells can be homogeneously and randomly distributed throughout a carrier matrix, and then infused into a scaffold. This is similar to the methods using fibrin gel to deliver cells to polyglycolic acid (PGA) scaffolds (Ameer *et al.*, 2002) and Matrigel to deliver cells into collagen sponges (Radisic *et al.*, 2003). The use of a hydrogel infusion cell delivery vehicle is very useful for slow attaching primary cells because with standard cell attachment-dependent cell seeding techniques, the probability of losing cells from a porous synthetic scaffold with a short incubation period is high. It has been shown that using collagen microsponges increases cell seeding efficiency (Chen *et al.*, 2004b). Collagen is chemotactic and capable of stimulating further deposition of more

matrix proteins (Kleinman *et al.*, 1982). Collagen also has the advantages of being a biological ECM and has a history of being used effectively in cardiovascular tissue engineering applications (Berglund *et al.*, 2003; Imberti *et al.*, 2002; Nasseri *et al.*, 2003; Nillesen *et al.*, 2007; Stegemann and Nerem, 2003a; Thomas *et al.*, 2003). We synthesize and use kangaroo tail tendon as a source of collagen due to the high collagen yield per animal compared to other species. Traditionally, yield of collagen is extremely variable and dependent on the animal species and the age of the animal. Very low yields from human skin to higher yields in young rat skin of up to 60% have been reported (Bazin and Delaunay, 1976) with the richest sources of collagen fibres being from the skin and tendons of adult mammals (Steven, 1976). However, if we consider total yield per animal tissue sample, especially in the face of animal ethics considerations, use of kangaroo tail is the most economical and high yield source. Also, use of relatively young animals is important as the proportion of insoluble collagen in most organs increases with age and can be affected by a number of physiological and pathophysiological situations (Deyl and Adam, 1976).

Figure 4 shows a porous 5wt%/vol poly DL-lactic-co-glycolic acid (P(DL)LGA) (75:25) scaffold with 53–106 µm pores that has been cut in half showing collagen fibres dispersed within the scaffold pores. Another benefit of using hydrogel cell delivery is that the hydrogel can also incorporate relevant growth factors to stimulate tissue-specific growth (Park *et al.*, 2007) as well as for cell delivery. Figure 5 shows human microvascular cells SV-40 HMEC-1 showing clear alignment within a cell/collagen hydrogel/scaffold system.

#### Optimization of system parameters

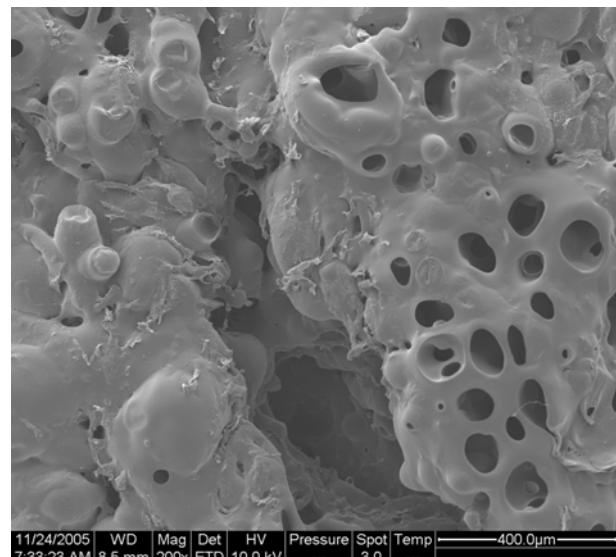
Mass transfer has been a serious problem for thick tissues that do not attempt to include some sort of vascular supply or inherent channel structures throughout the scaffold to



**Figure 4.** SEM of 4mg/mL collagen hydrogel infused 5wt%/vol P(DL)LGA(75:25) scaffold with 53-106 $\mu$ m diameter pores. Samples were processed from cryohistology. Black star (\*) indicates some of the collagen fibers from the dehydrated hydrogel.



**Figure 5.** Haematoxylin and eosin-stained SV-40 HMEC-1 within 4mg/mL collagen hydrogel infused within a porous 5wt%/vol P(L)LA scaffold 53-106 $\mu$ m diameter pores. Outlined regions show that there is clearly anisotropic cell alignment and aligned cell clustering. 4 week static culture.



**Figure 6.** Equine BMSC cultured on a porous anisotropic pore 5wt%/vol P(DL)LGA scaffold with ~200 $\mu$ m diameter pores. Even though the surface is populated with BMSC and it's matrix, there are clearly visible pore openings (black holes). 5 day static culture.

allow nutrients and wastes to diffuse. The use of macro-lumens and other similar channels is an inherent structural method to aid in creating better access to nutrients for cells within a scaffold (Kaihara *et al.*, 2000; Kannan *et al.*, 2005; Kim *et al.*, 1998b; Moldovan and Ferrari, 2002; Schantz *et al.*, 2005; Shin *et al.*, 2004; Vacanti *et al.*, 2003). With an optimal cell seeding and cell/scaffold culturing condition, such as dynamic cell seeding followed by bioreactor culture (Burg *et al.*, 2000), neovascularisation might be able to occur fast enough in highly porous scaffolds.

However there is a limit to this as there is an access gradient from the cells at the surface, which have the best access to nutrients, to the cells further away from the surface. However, it may be theorised that this may not be as big a disadvantage as it first seems. Since angiogenesis forms due to ischemia and nutrient/oxygen gradients in tissues, this nutrient/oxygen gradient in cell/matrix/scaffold systems may actually promote vessel in-growth and formation. The extent of mass transfer limitation can also be controlled to some extent by changing the concentration of the collagen hydrogel. Another method that we are testing is the use of anisotropic pore scaffolds which will minimize the tortuosity of the scaffold pore structure and therefore increase mass transfer. Figure 6 shows a scanning electron micrograph of equine BMSC cultured on one such anisotropic pore 5wt%/vol P(DL)LGA scaffold created using a TIPS method (Cao *et al.*, 2003; Cao *et al.*, 2006). Clearly seen are multiple black pores on the surface of the cell/scaffold construct. We are comparing the cell viability and distribution characteristics of this design with the standard highly interconnected and porous isotropic scaffolds. Another option for increasing mass transfer of oxygen could be to use cell culture media additives such as perfluorocarbon to enhance the supply of oxygen to cells (Radisic *et al.*, 2006) or the use of novel molecules for therapeutic angiogenesis (Sengupta *et al.*, 2004).

Optimization of the tools of tissue engineering used such as scaffolds, bioreactors and molecular and physical signalling is complex and requires a multidisciplinary approach and team of researchers and collaborators (Freed *et al.*, 2006; Mikos *et al.*, 2006). It is hoped that this current combinatorial approach will contribute to the research in bioengineered tissue vascularisation *in vitro*. One aspect that needs to be addressed further is the anisotropic nature of vascularisation and to design scaffolds and bioreactor systems that can mimic or nurture the potential to accommodate this anisotropy (Borselli *et al.*, 2007; Lanza *et al.*, 2006). These biomechanical influences will in turn affect the remodelling of the vascular structures (Jones *et al.*, 2006).

## Conclusions

The current trend in vascularising bioengineered tissues is the integration of various parameters that have been observed from *in vivo* biological research and *in vitro* bioengineering research. These include combining design parameters of highly porous macro- and micro-channelled polymeric scaffolds, the use of biological ECM matrices such as collagen hydrogels, microvascular cells co-cultured with other cells or growth factors, and culturing the cell/scaffold constructs in biomechanically dynamic environments. Microvascular cell co-culture with collagen hydrogel infusion in a highly interconnected and porous polymeric scaffold containing macro-lumens is one such integrated parameter system for creating a vascularized tissue. Benefits include the provision of a 3D matrix for cells to immediately adhere to within the scaffold, and gelling within a scaffold ensures cell entrapment within the scaffold. Potential drawbacks include a reduction of mass transfer ability to central regions of scaffold, but this factor may be beneficial given that angiogenesis occurs in response to ischemia. With a combinatorial approach, many growth parameters and interactions will need to be optimized before a true vascular analogue can be viably developed and maintained *in vitro* for tissue engineering. Finally, the ability to create a vascular analogue *in vitro* is very important to ensuring the viability and functionality of bioengineered organs, as is the capacity to connect to host vasculature upon implantation.

## Acknowledgements

This project was funded by a Discovery Grant from the Australian Research Council. Thanks to Ms Pankaj Godara and A/Prof. John Whitelock (University of New South Wales) for discussions on collagen processing, and Prof. Justin Cooper-White and Ms Sue-Ann Lim (University of Queensland) for discussions on scaffold designs and supply of anisotropic scaffolds.

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## Discussion with Reviewers

**V. Braunstein:** The authors mention rightly, that there seems to be a small range between desirable and undesirable ischaemia in engineered tissues leading to the stimulation of vessel ingrowth. Which systemic or local parameters could be helpful to have a more or less continuous monitoring of the viability of the engineered tissues?

**Authors:** At the moment, there seems to be no one optimal combination of cells, matrix or scaffold design for controlling vessel ingrowth and viability *in vitro*. Scaffold architecture parameters, such as porosity, pore size distribution, degree of pore interconnectivity, and scaffold pore system tortuosity, all influence the capacity for cell viability and nutrient medium mass transfer into a scaffold. We routinely monitor pH, glucose, lactate and cell viability using metabolic assays such as AlamarBlue. Using these ‘aggregate’ measures, and by controlling the architectural and culturing environment parameters, we aim to influence

formation of, for example, ischaemic gradients within the three-dimensional construct. Recent advances in imaging technology and fluorescent probes are also bringing the spatial visualisation of such phenomena closer to reality. Ultimately we would like to utilise these approaches to dynamically influence vessel ingrowth through, for example, temporal changes in nutrient medium composition, changing the magnitude of fluid flow within the tissue analogue, or altering the amount of dissolved oxygen in the medium. If we can quantify and correlate distinct physical growth conditions with the resulting effects on tissue growth, we may be on our way to defining the exact combination of parameters that can control vessel ingrowth *in vitro*.

**L. Koole:** Obviously, it is technically difficult to realise formation of arterial and venous blood vessels *in vitro*. Will there be perfusion? If so, with what? If not: will the tissues be capable of withstanding arterial pressure after implantation?

**Authors:** This system is designed to eventually be perfused through the nutrient channels. The extensive literature in this area indicates that perfusion is beneficial for proper “tissue” formation and cell alignment, with biomimetic pulsatile perfusion conferring enhanced mechanical properties on engineered blood vessels. The perfusion pressure implemented *in vitro* will depend on the type of tissue being created, since the distribution of pressure along the vascular tree strongly depends on the type of organ and its vascular topology. The compliance and elastic modulus of the supporting scaffold are important parameters here, but we have not investigated these at this early stage. Since we are essentially aiming to create a vascularized ‘block’ of tissue, we have a greater mass of scaffolding material available than would be the case for an engineered vascular graft for example, and this may offer some advantages in terms of more bulk material strength. To date we have cultured these constructs with normal cell culture medium such as MCDB-131 with growth supplements, but recognise that this needs to be optimised to take into consideration the changing needs of the growing tissue. A crucial stage will be the performance of the constructs when perfused with whole blood *in vitro*, since it is clearly essential to avoid thrombus formation when implanted.

**L. Koole:** How do the authors look upon the endothelium, i.e. the cells that will form the interface between the flowing

blood and the implant? Will there be a difference in arterial endothelium and venous endothelium prior to implantation, or differentiation only after implantation?

**Authors:** A functional endothelium is critical to the success of this approach for many reasons, not the least of which is the presentation of a non-thrombogenic surface to the flowing blood. We have seeded the constructs with microvascular endothelial cells both alone and co-cultured with other cell types. However, achieving spatial localisation to represent venous and arterial elements would be technically challenging. From a biomimetic standpoint, clearly fluid flow forces have a part in determining where arterial and venous endothelium start and end in the vasculature. If we can expose the tissue analogue to an appropriate gradient of force we may be able to form such a differentiated system *in vitro* before implantation. Whilst blood plasma is a Newtonian fluid, whole blood exhibits shear-thinning behaviour (i.e. viscosity decreases when shear rate increases) due primarily to red blood cell aggregation. It may therefore be necessary to use cell culture medium containing particles that mimic red blood cells, or use whole blood. That said we believe that it may be unwise and unnecessary to ‘overengineer’ the process of vascularisation. Rather, we aim to provide a template which can then be remodelled and adapted by the host tissues whilst maintaining functionality.

**L. Koole:** Do the authors have any information on the suturability of engineered tissues? In other words: can engineered tissue be “connected” to the arterial and venous vessels of the recipient organism, using standard anastomosis techniques? Is there really no risk that the (new or existing) blood vessels will tear at the suture site, or burst altogether?

**Authors:** Part of our original research aim was to create suturable vascularised tissue analogues. Whilst the porous PLGA materials used for the bulk scaffold clearly lack appropriate mechanical properties, our approach will be to incorporate suturable nanofibrous polyurethane mesh tubes at the anastomoses. As well as having mechanical properties designed to minimise undesirable outcomes such as anastomotic hyperplasia, these materials would degrade slowly or not at all, giving extra strength for suturing and time for host cell infiltration to reinforce the anastomosis. It may also be possible to seed this region with a high proportion of vascular smooth muscle cells to give more strength to the vessel during suturing.