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Biofabrication of Vasculature in Microphysiological Models of Bone

Ian T. Whelan, E Moeendarbary, David A. Hoey, Daniel J. Kelly.

Abstract

Bone contains a dense network of blood vessels that are essential to its homoeostasis, endocrine function, mineral metabolism and regenerative functions. In addition, bone vasculature is implicated in a number of prominent skeletal diseases, and bone has high affinity for metastatic cancers. Despite vasculature being an integral part of bone physiology and pathophysiology, it is often ignored or oversimplified in *in vitro* bone models. However, 3D physiologically relevant vasculature can now be engineered *in vitro*, with microphysiological systems (MPS) increasingly being used as platforms for engineering this physiologically relevant vasculature. In recent years, vascularised models of bone in MPSs systems have been reported in the literature, representing the beginning of a possible technological step change in how bone is modelled *in vitro*. Vascularised bone MPSs is a subfield of bone research in its nascency, however given the impact of MPSs has had in *in vitro* organ modelling, and the crucial role of vasculature to bone physiology, these systems stand to have a substantial impact on bone research. However, engineering vasculature within the specific design restraints of the bone niche is significantly challenging given the different requirements for engineering bone and vasculature. With this in mind, this paper aims to serve as technical guidance for the biofabrication of vascularised bone tissue within MPS devices. We first discuss the key engineering and biological considerations for engineering more physiologically relevant vasculature *in vitro* within the specific design constraints of the bone niche. We next explore emerging applications of vascularised bone MPSs, and conclude with a discussion on the current status of vascularised bone MPS biofabrication and suggest directions for development of next generation vascularised bone MPSs.

1. Introduction

Despite the substantial contribution of animal models to drug discovery and basic biological research, their shortcomings as analogues of human (patho)physiology are

now well recognised [1, 2]. In many cases, animal models are unsuitable analogues of human biology. For example, the human immune system [3, 4] and blood brain barrier [5, 6] cannot be modelled accurately with existing animal models. The poor predictive ability of such models, combined with their complexity and high development costs, has motivated the search for alternative approaches to model human biology. Animal testing is the gold standard in bone research, yet it is recognised that further development of *in vitro* systems to replace and augment animal models is needed [7, 8]. The routine use of animals in biomedical and engineering research is under increasing scrutiny, and much effort is now focussed on reducing animal numbers in research [9]. Due to the relative inaccessibility of primary human bone cells, the state of the art *in vitro* systems used to probe the mechanisms of bone physiology and pathology are typically 2D systems with immortalised cell lines derived from murine sources. Thus, the field of *in vitro* bone research stands to gain from development of more physiologically relevant human bone models.

MPSs are an emerging technology that involve the biofabrication of human organ systems at the microscale. These systems are similar to traditional cell culture systems in terms of ease of use and experimental control, but can add biological complexity in the form of multiple cell types, complex tissue geometry, fluidic coupling of devices, mechanical stimulation, and vascularisation. Bone is a highly complex organ, in which many of these aforementioned parameters are critical to its physiology. For example, osteocytes, the cells that comprise greater than 90% of cells in bone [10], are highly mechanically sensitive [11], and transduce mechanical stimuli to coordinate bone remodelling; a process disrupted in prominent diseases such as osteoporosis. Additionally, bone vasculature is tightly integrated in endochondral bone formation [12, 13], and bone tissue is a common secondary site for tumour cells to extravasate from the vasculature in metastasising breast and prostate cancer [14, 15]. Thus, the additional complexity offered by MPS systems may facilitate engineering more physiologically relevant bone models, and potentially lead to significant discoveries about the fundamentals of bone physiology and pathology.

Vasculature plays a key role in many (patho)physiological processes in bone (Figure 1). Vascular invasion is a critical step in endochondral ossification (Figure 1A), the process by which most bones develop prenatally, and grow and repair postnatally, as it drives the conversion of cartilaginous template into new bone. It is still not fully

understood how vasculature drives this process, but insights into how it can be regulated could provide treatments for non-unions, chondrodsplasias and osteochondrosis. Bone remodelling is a key process where osteoblasts, osteoclasts and osteocytes maintain bone tissue health by continuous deposition and resorption. It is estimated that the whole skeleton turns over every 10 years [16], and this intricate process becomes dysregulated in conditions such as osteoporosis [17], Paget's disease, and renal osteodystrophy [18]. We now know that vasculature plays a key role in bone remodelling by supplying the key growth factors and precursor cells to the bone remodelling unit [19] (Figure 1B). Additionally, the vasculature plays host to the stem cell niche in bone marrow, which maintains the naïve phenotype of stem cells, which are important to many bone functions, including regeneration [20] (Figure 1C). More recently, bone has been shown to play a significant role in glucose handling in humans [21], where undercarboxylated osteocalcin is released by bone resorbing osteoclasts and released into the circulation to exhibit endocrine effects on the testes and pancreas (Figure 1D). Interestingly, dysregulated glucose handling in diabetes mellitus is associated with impaired blood flow, decreased vessel supply in long bones of rats, and has also been associated with increased risk of fractures in humans [22]. Finally, Metastasising cancers of the breast and prostate have an affinity for bone tissue, with post mortem examination showing 70% of patients have bone metastases [23]. While still not fully understood, these findings suggest that bone tissue creates a niche that favours metastatic colonisation for circulating tumour cells (Figure 1E). Common to all of the above is the central role that the vasculature plays in these biological processes in bone, yet it is poorly represented or absent from the majority of *in vitro* models of this organ.

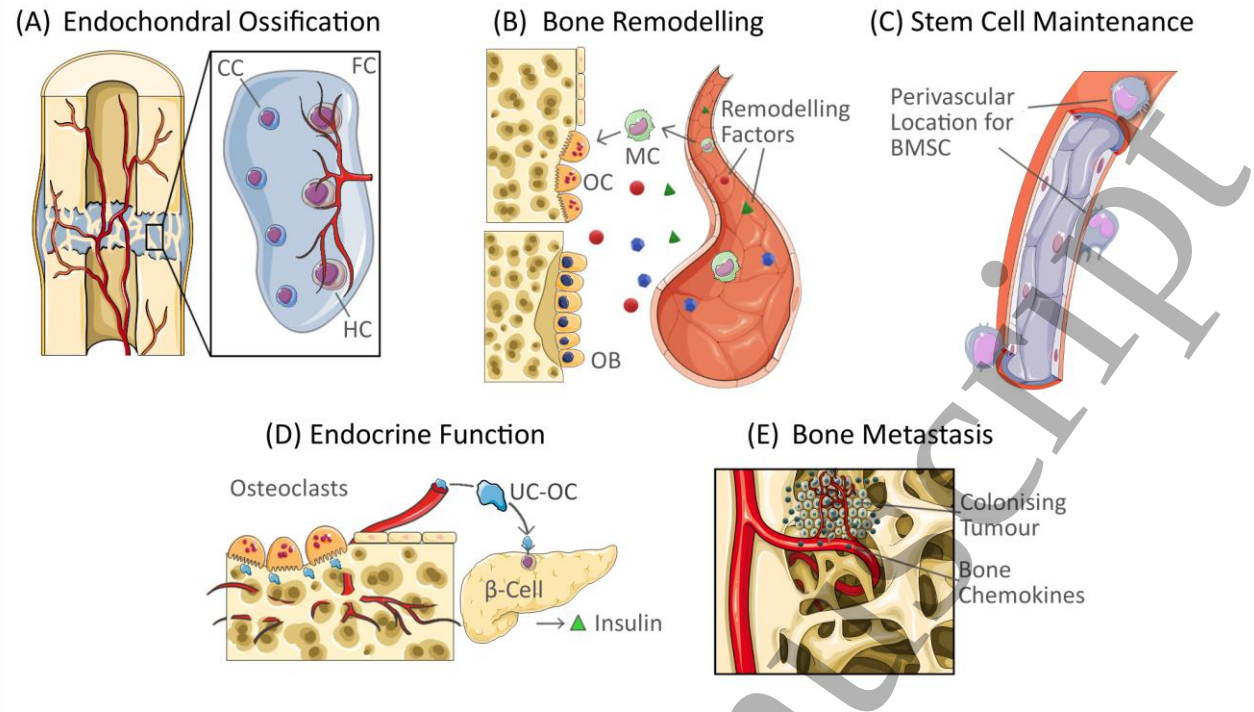

Figure 1.1: Role of Vasculature in bone physiology and pathology. (A) Endochondral ossification during fracture healing. (B) Bone remodelling is supported by vasculature; supplying necessary growth factors and precursor cells to sustain remodelling. (D) Osteocalcin, a bone derived hormone, is activated and released from mineralised tissue into the circulation through de-carboxylation by resorbing osteoclasts. (E) Bone facilitates metastatic colonisation through secretion of chemokines that attract circulating tumour cells. CC-Chondrocyte, FC-Fracture Callus, HC-Hypertrophic Chondrocyte, MC-Monocyte, OC-Osteocyte, OB-Osteoblast, UC-OC-Uncarboxylated Osteocalcin

The importance of incorporating vasculature in *in vitro* models is well recognised [24, 25] and over the last 10 years, numerous different approaches for fabricating microvascular networks in MPSs have been described [26]. However, engineering vascular networks within the constraints of a specific organ niche is significantly more challenging, and this is particularly the case In the context of bone [27, 28]. In light of this, the proceeding section describes the key considerations for developing physiologically relevant vasculature within the bone nice.

2. Considerations for prospective vascularised bone MPSs

The vascular component of a bone MPS can be engineered by approaches that largely fit into one of two categories; Top-down engineered vessels and bottom-up self-assembled vessels. Top-down engineered vasculature is achieved by fabricating pre-patterned lumen structures within MPS devices and subsequently coating the luminal surface with endothelial cells. A number of strategies exist for engineering vasculature

by such methods, and have been reviewed elsewhere [29]. In contrast, vascular self-assembly relies on creating conditions to allow endothelial cells to form physiologically relevant vascular networks, typically within a hydrogel, and is the most common method in MPS systems as this vasculature is more reflective of the *in vivo* condition. This can be achieved through vascular invasion into the gel in response to an angiogenic gradient in a process analogous to angiogenesis, or by spontaneous formation of vasculature in a process analogous to vasculogenesis. Naturally, the choice of which methods to use depends on the application; geometrical control of vessels is important for regulating fluid flow and shear stresses, or recreating the geometry of haversian canal within osteons. However, self-assembled vasculature is a more physiologically relevant analogue, and is the ideal endothelial niche to represent vasculature in these models. Thus the proceeding sections are a discussion of the pertinent factors in engineering this self-assembled vasculature in a bone context.

2.1 Cells

Endothelial cells (ECs) are a heterogeneous population, with phenotypes that reflect their *in vivo* niche. Bone microvascular ECs would naturally be an ideal candidate for bone models, however while a bovine clonal EC line has been reported [30], it has not been used extensively, and no cells from human origin exist. However, it must be noted that bone ECs have shown to be sensitive to hormones involved in bone homeostasis, such as parathyroid hormone (PTH), where ECs from other sources have not [30], and bone ECs have been shown to express estrogen receptors, and to proliferate and show inhibited PTH responsiveness when treated with estrogen [31]. Despite the case for bone specific ECs, they are not used extensively, though modelling specific biological processes may require a specific bone derived EC.

At present, commercially available primary cell sources are most commonly used for the endothelial component of MPSs (Table 1), including human umbilical vein ECs (HUVECs) and human microvascular ECs (HMVECs), and endothelial progenitor cells (EPCs). In addition, while much success in engineering microvascular networks has been reported using these primary cells, there has been increased interest recently on the use of induced pluripotent stem cells (iPSCs). This section aims to compare these

cell types in the context of engineering the vascular component of a vascularised bone MPS.

2.1.1 Vasculature: Endothelial Cells

HUVEC / HMVEC

It is well documented that primary human ECs are not a homogeneous cell source, but vary depending on their origin [32-35]. A subset of ECs, namely HUVECS and HMVECs, are the two most ubiquitous primary EC types used in vascular network biofabrication. HUVECs (see Table 1) have been used almost exclusively as the cell type for engineering vasculature within MPS. However, HMVECs from various origins (brain, lung and skin) are also used in vascular research for studying angiogenesis [36], metastasic intravasation [37], and engineering vasculature [38, 39]. Despite differences between ECs in general, evidences suggest that these two particular EC types may be functionally similar. For example, HUVECs and HMVECs behaved similarly, in terms of cell migration and morphogenesis, when subjected to various chemokines [40]. HUVECs and HMVECs also showed similar contractility and matrix invasion functions when seeded in a collagen lumen [38]. Furthermore, both cell types have also been shown to deposit similar amounts of basement membrane protein [41]. However, conflicting evidence arises in the context of barrier function. HMVECs were shown to display more continuous ZO-1 and occludin staining, higher transendothelial electrical resistance (TEER) and lower permeability compared to HUVECs [42]. In conflicting reports, HUVECs have exhibited lower permeability compared to HMVECs [37], and cerebral ECs [43]. A possible explanation for this inconsistency might be that in those conflicting studies, HMVECs were isolated from different sources of brain and skin.

Endothelial Progenitor Cells / Endothelial Colony Forming Cells (EPCs/ECFCs)

EPCs are circulating progenitor cells that can differentiate into all cell types of the capillary niche [44], making them an exciting prospect for vascular network biofabrication. EPCs have been historically isolated and characterised using a variety of methods such as molecular sorting, adherence enrichment, and adherence depletion [45], and only recently have a set of molecular and functional requirements been established to isolate and characterise putative EPCs [46]. EPCs are colony forming cells that are restricted to the EC lineage, as evidenced by endothelial marker

expression; ability to undergo 30 population doublings over 60 days of *in vitro* culture; form lumenised capillaries *in vitro*, and can anastomose to host vasculature *in vivo* [46]. Colony formation was a key development during the search for EPCs, thus the term endothelial colony forming cells (ECFCs) was coined and represents EPCs isolated and characterised using this method. EPCs have been used as an EC source to fabricate vascularised tissue in a number of reports [47-49], however, their use as the endothelial component in MPSs is limited. There is one report that used blood outgrowth ECs (BOECs), a less purified subset of EPCs, as a patient-specific cell source for modelling thromboinflammation. The authors found vessels engineered using these cells from diabetic patients exhibited a pro-thrombotic and pro-inflammatory phenotype which was not evident with cells from healthy patients [50]. EPCs may prove to be a valuable patient specific cell source for modelling specific conditions such as thromboinflammation in diabetes. However the lack of use of these cells within MPSs, the additional steps required to isolate and purify from a venous draw, and the variability that comes with lack of standardisation in isolation and characterisation [46, 51] may limit their application until these issues are resolved.

Induced Pluripotent Stem Cells

Recently, attention has been focussed on the application of iPSCs in tissue engineering and disease modelling. In the latter case, iPSCs represent a patient specific pluripotent cell source to study disease progression in multiple cell types and tissues, a paradigm also applicable to MPS. Aside from their self-renewing capacity, iPSCs retain the genetic backgrounds of the patients from which they were derived and may serve as a disease specific cell source. ECs derived from iPSCs (iPSC-ECs) have the ability to form vascular networks *in vitro* and *in vivo*, and display the molecular signature of mature vessels [52, 53]. While these results are promising, only recently a comparison between iPSC-ECs and primary ECs has been conducted [54]. The report compared isolated HUVECs, commercially available HUVECs, and two iPSC-EC cell sources and found that sprouting of iPSC-ECs was significantly attenuated compared to HUVECs, with the study citing reduced MMP-9 expression as a possible mechanism. iPSC-ECs also proliferate more slowly compared to ECs, which may hinder their utility in large scale MPS systems [55]. Finally, the endpoint of iPSC-EC differentiation has yet to be standardised, thus reported iPSC-ECs function in literature

likely represent the function of a range of iPSC-EC phenotypes, depending on the specifics of the study in question.

2.1.2 Parenchyma: Bone Cells

The formation of vasculature in a vascularised bone MPS requires a support cell. In many applications, fibroblasts have been the support cell of choice as they readily facilitate formation of vascular networks and can be cultured up to high passages. However, the formation of vascular networks in a bone niche requires a more tissue relevant support cell type. The key parenchymal cells in mineralised bone tissue are the osteoclasts, osteoblasts and osteocytes, with bone marrow stromal cells (hBMSCs) the resident stem cell precursor of the latter two. These cells make up the bone remodelling unit and work in tandem to maintain bone homeostasis.

Osteoclasts

The exact relationship between vasculature and bone resorbing osteoclasts is not well understood. There is some evidence that suggests suppression of osteoclast formation with osteoprotegerin, an osteoclastogenesis inhibitor, in bone explants will dose-dependently inhibit angiogenesis [56]. Additionally, a recent study has shown that a subset of osteoclasts, the vessel associated osteoclasts, regulate anastomoses of type H vessels, the vessels found in the bone metaphasis, during growth plate resorption during bone growth [57]. Despite this, no *in vitro* studies have shed light on how osteoclasts specifically effect vascular network formation *in vitro*. This is surprising given that the effects of osteoporosis therapeutics typically target osteoclasts [58], but also effect angiogenesis and potentially cause osteonecrosis [59]. A number of human osteoclast analogues have been used in *in vitro* culture systems; $C14^+$ Mononuclear cells from peripheral blood can be stimulated with RANKL and M-CSF to yield a population of osteoclast like cells [60], and osteoclast populations can be isolated from the non-adherent cells in bone marrow [61].

Osteoblasts

Osteoblasts line the bone surfaces and contribute to bone formation through secretion of the organic components of bone tissue including predominantly collagen type I, proteoglycans, glycoproteins and γ-carboxylated proteins [62]. Osteoblast function is dysregulated in conditions such as diabetes mellitus [63] and osteoporosis [64]. *In vitro* investigation of osteoblast function typically relies on the murine derived MC3T3 and

MLO-A5 cell lines [65]. Human osteoblasts are far less common, but they are commercially available and protocols exist for their isolation [66]. Primary osteoblasts have been found to promote EC proliferation and formation of vessel structures [67]. Additionally, Ma *et al.* have shown that primary human osteoblasts facilitate vessel formation in HUVECs in 2D culture [68]. Thus, though unproven, human osteoblasts may be able to supply the required factors for vascular network morphogenesis.

Osteocytes

Osteocytes comprise over 90% of the cells in mineralised bone tissue and are a key orchestrator of bone function. Osteocytes are believed to be responsible for transduction of mechanical signals [69], orchestration of bone remodelling [70], and endocrine regulation of distant organs [71]. Thus, much of reported *in vitro* bone models attempt to recreate osteocyte function. The conditioned media from the osteocyte cell line MLO-Y4 has been shown to support vascularisation *in vitro*; having effects on EC proliferation and network formation [72, 73]. Primary human osteocytes can be obtained by collection of late stage cells in serial digestion of trabecular bone in a chelating agent and collagenase [74]. However, it is difficult to yield large numbers of these cells, and they tend to de-differentiate in culture. As yet, no human analogue of the osteocyte exists, and development of such a model will be key for *in vitro* vascularised bone research.

Bone Marrow Stromal Cells

Bone marrow derived multipotent stromal/stem cells (BM-MSCs) are the progenitors of both osteoblasts and osteocytes. They are commonly used in bone applications as they can be osteogenically committed either directly, or with initial chondrogenic priming before hypertrophic induction. To complement this, BMSCs have been widely studied, are more available than other human bone cell types, and have proven to facilitate vascular network formation. These, along with the fact that BMSCs are believed to be the precursors of osteoblasts and osteocytes, and originate from mural cells that induce network stabilisation, has made BMSCs the canonical cell type for generating bone in MPS systems.

Undifferentiated BM-MSCs facilitate vascular network formation in co-culture with ECs, and tend to differentiate toward a mural cell like phenotype; expressing α-SMA and migrating to the perivascular space [75, 76]. These traits may be a promising

means to promote vessel maturity in bone MPSs, as co-culture with pericytes has shown improved basement membrane production [77], decreased vessel permeability [77-79] and inhibition of vessel regression [80]. Upon differentiation, BM-MSCs exhibit a more osteoblastic phenotype, and this has been achieved in vascularised MPS systems by pre differentiating BM-MSCs in monolayers before MPS co-culture with ECs [81, 82]. In this case BM-MSCs retain their osteoblastic phenotype; expressing osteocalcin and ALP. A key consideration for forming vascular networks using BM-MSCs is the cell ratio; high relative numbers of BM-MSCs relative to HUVECs (1:2) will form vascular networks, but will require additional VEGF supplementation and 2D cell coverage in the media channels to facilitate limited perfusability [75]. However, the same group discovered subsequently that the relatively high number of MSCs used in these studies (1:2 BM-MSC:EC), a common ratio used with fibroblasts, was prohibitive of perfusability, and using a decreased relative number of BM-MSCs (1:10) resulted in the formation of perfusable networks [81, 83]. The reason that BM-MSCs need to be in relatively low numbers to support perfusable vasculature is still unknown. Perhaps, as BM-MSCs are believed to be derived from pericytes [84], they may possess the documented stabilising nature of pericytes, such as their abrogating effects on VEGF [85].

One critical consideration for support cell selection is their effect on the proteolytic behaviour of ECs. Comparisons have been undertaken between bone marrow derived MSCs (BM-MSCs), adipose derived MSCs (AD-MSCs) and normal human lung fibroblasts (NHLFs), and their effects on the vasculogenic process *in vitro* (Figure 3.1). In a 3D fibrin matrix co-culture with BM-MSCs, ECs critically rely on membrane bound metalloproteinases, specifically MMP-14 (MT-MMP), with MMP inhibition halting EC sprouting [86, 87] (Figure 3B). In contrast, with AD-MSCs and NHLFs, EC sprouting proceeds despite MMP inhibition. NHLFs and AD-MSCs promote ECs to remodel their ECM during angiogenesis through both MMP and plasminogen activator / plasmin axis [88], requiring inhibition of both programs to halt sprouting. Thus, the choice of support cell type may influence the proteolytic mechanism by which ECS form vascular structures, which is a key consideration for both support cell and ECM selection, or use of inhibitors of these proteolytic processes in these systems.

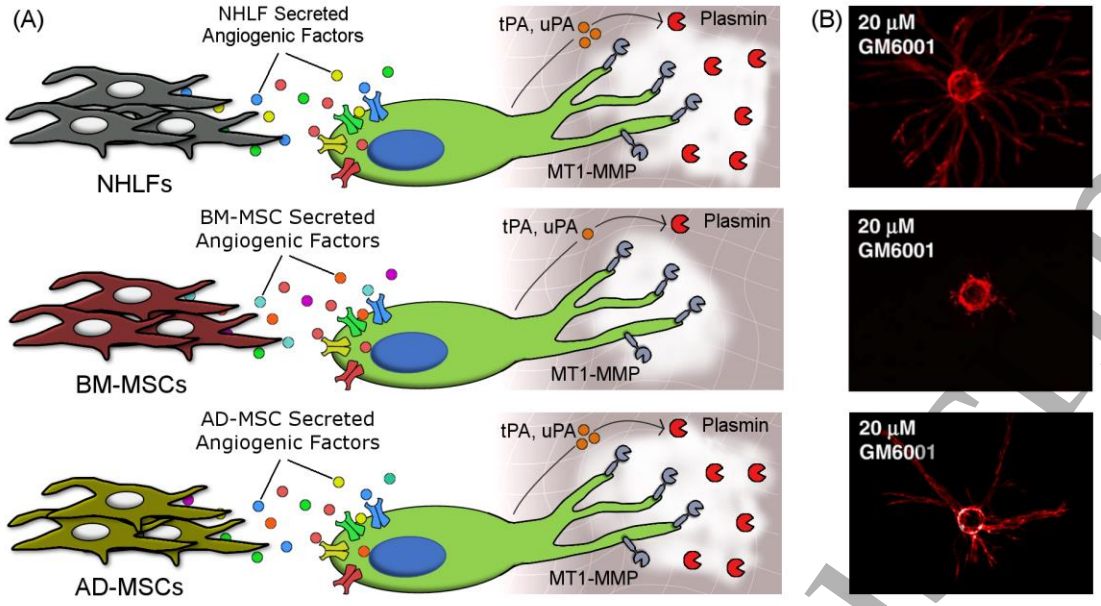

Figure 3.1: Different Support Cell types cause ECs to remodel their ECM using different proteolytic mechanisms. (A) NHLFs and AD-MSCs are proteolytically plastic and can invade fibrin matrices when either MMPs or the PA/Plasmin axis are inhibited. BM-MSCs rely heavily on membrane bound MMP to degrade fibrin. **(B)** Broad spectrum inhibition (GM6001) of MMPs prevent BM-MSCs from invading fibrin gels [86, 88].

2.2 Soluble Factors

In vivo, ECs lining stable blood vessels remain quiescent due to a balance of pro-angiogenic and anti-angiogenic factors [89, 90]. Vessel growth (pathological or otherwise) or regression is triggered when this balance is changed, and either pro- or anti-angiogenic stimuli dominate. *In vitro*, ECs are grown and maintained in culture media that is typically supplemented with a number of these soluble angiogenic factors. Commercial EC growth media (EGM) can be broadly categorised into EGM and EGM-2. EGM-2 is low serum media typically supplemented with several angiogenic factors, such as vascular endothelial growth factor (VEGF), hydrocortisone, epidermal growth factor (EGF), Insulin-like growth factor (IGF), Ascorbic Acid (AA), basic Fibroblast Growth Factor (bFGF) and hepatocyte growth factor-B (HGF-B). EGM is intended for rapid EC growth and is also used as the medium to facilitate vascular network formation in 3D. In bone, osteogenesis and angiogenesis are inherently coupled, thus a number of bone cells secrete factors that that effect vascular network formation

2.2.1 Angiogenic Factors in Bone

A number of angiogenic factors that regulate bone physiology *in vivo* may be of particular importance when recreating bone physiology *in vitro*. Evidence suggests

VEGF is a key regulator of angiogenesis in bone tissue and differentiating osteoblasts [91], osteocytes [92], and bone ECs [93] have been shown to express this chemokine. FGF-2 is also expressed by osteoblasts [94], and induces osteoclastogenesis [95] and osteoclastic bone resorption [96]. More interestingly, a number of bone associated factors have been shown to have potent pro angiogenic effects. Bone morphogenetic proteins (BMPs) are crucial proteins in the development and maintenance of skeletal tissues. BMP-2 has been shown to induce angiogenesis in endothelial progenitor cells [97], and has also been shown to be a specific promotor of angiogenesis in developing cancers [98, 99]. BMP-7 has been to promote angiogenesis in a chick chorioallantoic membrane [100], and BMPs 2, 4 and 9 have also shown to have pro angiogenic effects [101].

Receptor activator of nuclear factor kappa-B ligand (RANKL) and osteoprotegerin (OPG) are key factors in regulating bone resorption though their regulatory effects on osteoclasts, but also have different regulatory effects on angiogenesis. OPG, aside from its role in bone, is also produced by ECs, and is released when ECs are stimulated by TNF-α, suggesting OPG has a role in regulating inflammation [102]. OPG also maintains EC viability by blocking apoptosis [103], and induces angiogenic sprouting in an aortic ring model [104]. OPG has also been shown to stimulate colony formation in ECFCs [105]. The role of RANKL, the canonical ligand for OPG in bone, is less understood, as conflicting evidence exists on its angiogenic properties. RANKL was found to inhibit EC proliferation and angiogenesis [104] *in vitro*, but has also been show to promote angiogenesis *in vivo* [106] and has also been shown to have a role in promoting EC survival [107]. In summary, osteoclast inhibitor OPG appears to facilitate vessel formation, while the effects of its canonical ligand, RANKL, are less certain.

2.2.2 Effects of osteoinductive supplements on angiogenesis

Where required concurrently driving angiogenesis and osteogenesis of endothelial and mesenchymal precursors *in vitro* has proven challenging as it requires integrating the soluble factors required for each purpose. Osteogenic medium, for osteoblast and osteoblast precursors is typically high or low glucose essential medium supplemented with 10% FBS, 10-100 nM dexamethasone, 10mM β-glycerophosphate, and 10-50μg/ml ascorbic acid. The level of serum in osteogenic medium is higher than that of EGM (10% vs 2-5%). This is a key consideration, as serum levels are mediators of

tube formation. For example, serum levels are used as controls in Matrigel tube structure assays; low serum (5%), low supplement negative controls and high serum (10%) supplemented with FGF-1 and FGF-2 are used to decipher pro and anti-angiogenic activity [108]. Additionally, some evidence suggests that very high serum medium (20%) can prematurely induce EC senescence [109]. Dexamethasone is a synthetic analogue of the natural glucocorticoid hydrocortisone, used in EGM, and comparatively has a much higher affinity for glucocorticoid receptors [110]. Hydrocortisone is used to increase EC sensitivity to EGF [111], while excessive stimulation of glucocorticoid receptors has been shown to cause oxidative stress in ECs [112]. β-glycerophosphate is a phosphatase inhibitor and phosphate ion source for the formation of calcium phosphates during osteogenesis. To date, there is no evidence of adverse effects of β-glycerophosphate or phosphate ions, at cell culture relevant levels, on ECs. Finally, ascorbic acid, a vitamin and cofactor required for proper collagen synthesis [113], is required for both media. These observations would suggest that osteogenic differentiation and endothelial vascular morphogenesis can occur concurrently *in vitro.* However, concurrent differentiation of hBMSCs and vascular network formation in 3D has yet to be reported. In 2D, a range of optimised media have been reported for co culture of hBMSCs and ECs, but these experiments rarely evaluate both angiogenic and osteogenic outcomes [114, 115]. Thus, an optimal media formulation has yet to be realised that can drive simultaneous vascular network formation and bone matrix deposition in 3D.

2.3 Extracellular Matrix (ECM)

The ECM plays a central role in the formation of microvascular networks. During angiogenesis or vasculogenesis, the ECs exert pull-push forces on their surrounding ECM while cleaving and remodelling their environment as they migrate towards an angiogenic stimulus. The ECM can facilitate or hinder these processes; thus optimisation of ECM parameters is crucial for successfully vascularising a bone model. Engineering bone brings with it its own ECM and culture requirements, which adds an additional layer of complexity. Table 1 lists the matrix parameters used for constructing microvascular networks in MPS devices in both bone and applications in other organs, and will be referred to throughout the proceeding sections.

2.3.1 Matrix Materials

Natural biodegradeable materials are the most commonly used ECM analogues for forming vascular networks in MPS applications (Table 1). Fibrin and collagen type I (herein referred to as collagen) have been used almost exclusively in the published literature, and are deemed the gold standard material by the American heart association for 3D *in vitro* evaluation of vascular biology [116]. Fibrin gels are typically fabricated using a final fibrinogen concentration between 2-10 mg/ml, as circulating levels of fibrinogen in human blood are of this magnitude [117, 118]. ECs express urokinase plasminogen activator (uPA) during angiogenesis; a key driver of fibrin degradation. In addition to fibrin's degradeability, its degradation products, specifically fibrin fragment E, are generated during degradation and have potent angiogenic effects [119]. As fibrin matrix is canonically involved in acute healing, it is not present in appreciable levels in healthy bone tissue. However, fibrin is abundant at the site of bone fractures [120], and therefore may serve as an ideal material for engineering models of bone fracture healing. In addition, fibrin is becoming increasingly common in engineering bone implants for regeneration [121].

Collagen is another candidate ECM material that has been used for vascular network self-assembly in MPS systems [55], and is used in established angiogenesis assays such as the aortic ring assay [122]. Collagen is the primary structural protein in bone, and harbours the bone apatite crystals within the gap zones of the striated collagen fibrils [123]. In *in vitro* systems, acid extracted atelocollagen from rat tail is typically used, and when brought to within physiological range for pH and temperature, will undergo fibrillogenesis and form a gel. Conditions under which collagen gels are formed; such as pH, temperature, and ionic strength, effect the physical properties of the resulting gel such as mechanical strength, turbidity and pore architecture [124]. While collagen gel formation is thus sensitive to gelation conditions, studies that show these effects often use gelation conditions that exceed the practical boundaries of cell culture (eg gelation for 48 hours at 4˚C or gelation at pH 10). Despite this, ECs have been shown to be sensitive to alterations in collagen gel properties; collagen matrices with aligned fibre architecture enhances Collagen IV, a key basement membrane protein, and lumen formation [125]. ECs have also shown to decrease lumen size and density in collagen gels of increasing weight fraction up to 2% w/w [126].

Collagen 1 is ubiquitous in bone research as it is the subject of metabolic and catabolic activity of the key bone cells, and the environment in which they reside *in situ*. For example collagen 1 peptides act as chemoattractants for monocytes, the osteoclast precursor, suggesting a role for collagen 1 fragments in osteoclast recruitment [127], and mineralized collagen fragments from the ECM promote osteoclast differentiation [128]. Osteoblasts are anchored to the bone surfaces and their differentiation is induced by the integrins activated upon collagen binding [129, 130]. Osteocytes reside in a collagen 1 rich matrix, and most evidence suggests that this 3D biomimetic matrix is superior for maintenance of osteocyte phenotype and genotype in *in vitro* culture [131, 132]. Like osteoblasts, BMSC osteogenic differentiation is also induced and enhanced when cultured in collagen gels [133], or with gels that mimic collagen motifs for specific integrin activation [134]. Thus, collagen is a very applicable material for engineering vascularised bone MPSs.

Composite blends of both collagen and fibrin may be an ideal material for generating vascularised bone. Rao *et al* compared collagen, fibrin and blends of both polymers [76], and suggested that a 40/60 mass ratio of collagen/fibrin was optimal for vascular network formation, with total network formation increasing proportionally with weight fraction of fibrin in the blend. This study, and others [135-137] additionally found that increasing matrix density (by increasing polymer concentration) impedes angiogenesis; resulting in shorter, thicker, and slower-growing sprouts. ECs change their sensitivity to VEGF depending on the elasticity of their substrate [138], which may partially account for this observation. However, there is also evidence to suggest these effects can be somewhat abrogated by introducing a supporting cell type into these denser matrices [137]. Critically, these composite gels have additionally been shown to support osteogenesis [139].

Natural materials such as fibrin and collagen are used throughout the literature as the canonical ECM analogues for creating vascular structures in MPS applications However, an ideal ECM analogue would not have the natural donor variation inherent in biologically derived materials. While hydrogels based on synthetic polymers such as polyethylene glycol (PEG) [140], Polyethylene oxide (PEO) [141], and polyvinyl alcohol (PVA) [142] have been us, they are less prevalent in applications requiring vascular network formation or osteogeogenesis within MPSs. Thus, it is likely that the availability and ease of use of the gold standard natural polymers outweighs any

drawbacks of natural biological variation in current MPS application. Despite this, PEG hydrogels have been used to create vascular networks within MPS by modification with MMP degradable crosslinks. These materials have been used with HUVECs [143], and iPSECs [144], to successfully form vascular networks, and may be promising materials for vascularised bone engineering.

2.3.2 Matrix Mineral Functionalisation

When building bone models, a natural consideration is the incorporation of mineral into the matrix. Various calcium based ceramics are typical in bone tissue engineering as they support osteogenesis [145] but also provide mechanical support for load bearing [146]. In a vascularised bone MPS, ECM mineralisation must also not be detrimental to the formation of vascular networks. Jusoh *et al.* showed that hydroxyapatite can be incorporated into fibrin gels within a MPS and that optimal concentration for vascular network formation was at 0.2% or 20mg/ml [147], and additionally found non-uniform hydrogel formation at concentrations exceeding 40mg/ml. In a more general bone tissue engineering context, HDMECs cultured on various bioceramics formed vascular networks but required support from bone derived cells [148]. Chen et al. have also found that the particular calcium phosphate chemistry is an important consideration; with increased relative amounts of β-tricalcium phosphate enhancing neovascularisation *in vitro* and *in vivo* [149]. In addition to the phases present in calcium phosphates, the materials can be doped with trace elements found in bone tissue. Ions such as strontium [150], magnesium [151], copper [152] and silicon [153] have been shown to have pro-angiogenic effects. Thus, ECM functionalisation with bone-like mineral components can be achieved without impeding vascular network formation.

2.3.3 Engineered cell-matrix interactions

Synthetic hydrogels hold promise for realising reproducible and repeatable vascularised bone models. However, such materials would require mimicking the favourable properties of natural materials such as collagen and fibrin. One approach to engineering such materials is to engineer matrix with an optimal ligand presentation to support vascular formation and osteogenesis. In a vascular context, integrins are heavily involved in EC growth, survival and migration in angiogenesis [154]. In fibrin gels, $\alpha_v\beta_3$ and $\alpha_5\beta_1$ activation has been shown to be necessary for lumen formation in

3D [155, 156], and these same integrins have been shown to regulate EC invasion [157]. Additionally, $\alpha_v\beta_5$ is also a key integrin involved in EC invasion and differentiation in these same gels [158]. Currently, synthetic hydrogels are most commonly functionalised with an ECM mimicking peptides for biocompatibility and to allow cell adhesion. This is typically the Arg-Gly-Asp (RGD) peptide sequence, derived from fibronectin, and accounts for 89% of the cell adhesion motif of choice for synthetic ECM materials [159]. While the RGD motif can facilitate endothelial attachment and sprouting [160], networks are typically less interconnected and patent, thus its use is far less than that of natural biomaterials. However, evidence suggests that using integrin activation as biological cue in addition to a means to facilitate cell attachment, may be a promising strategy for vascular network engineering. Specifically, with the right combinations of integrins, ECs can be directed to develop functional vasculature [161]. Particularly, Li *et al.* demonstrated that modifying fibrin with engineered fibronectin that preferentially binds $\alpha_3/\alpha_5\beta_1$ or $\alpha_v\beta_3$ promotes remarkably different vascular phenotype; with α3/α5β1 binding gels facilitating formation of organised space filling, and functional vasculature both *in vitro* and *in vivo*.

Integrins are also critical for supporting osteogenesis. Though many integrins are expressed by skeletal cells [162], only few are known to support bone formation. $\alpha_5\beta_1$ is one such integrin, supporting osteoblast proliferation, differentiation and survival [163]. In addition, this integrin has been shown to be critical in the anabolic effects of mechanical loading, and its expression is downregulated when loading is absent [164]. Furthermore, agonists of the $\alpha_5\beta_1$ have been shown to induce osteogenesis in hBMSCs. [165]. Thus, a matrix engineered to express ligands for the key integrins involved in osteogenesis and angiogenesis could be a promising approach to engineer synthetic matrices for vascularised bone MPS applications.

ECM selection is critical for concurrently supporting vascular network formation and osteogenesis. To date the MPS models most relevant to generating vascularised bone have selected ECMs based on facilitating vascular network formation (Table 1). Generally, fibrin and collagen matrices are used in low concentrations to facilitate EC migration. Development of vascularised bone MPSs will require further development of matrices using the principals outlined above, to support osteogenesis as well as network formation.

2.4 Mechanical Environment

The influence of mechanics is a heavily researched area in bone biology [166], and different modes of mechanical stress are commonly applied to bone cells to investigate mechanotransduction. It is likely that vascularised bone models will require a mechanical component to appropriately model many bone physiological processes. In such cases, the vascular and bone component of a vascularised bone MPS will be concurrently under mechanical strain. It is therefore prudent to consider how the mechanical environment influences both processes.

2.4.1 Compression / Tension

Compression and tension are often used as a means to simulate the mechanical environment of loaded bone as a strategy to improve engineered bone tissue and for probing mechanosensation of bone cells [167]. In an MPS context, compression has been used in a model of cartilage compression [168], and thus is a realisable means of incorporating mechanical function into a vascularised bone MPS. Other applications such as gut [169] and lung [170] MPSs have cells cultured on flexible membranes that are stretched in tension under actuation from vacuum pumps to simulate both peristalsis and breathing in their respective applications. Such a system could conceivably be used to incorporate mechanical signals into a vascularised bone device. While the effects of mechanical signals such as these have been extensively studied in MSCs [167], osteoblasts [171] and osteocytes [172], comparatively little is known about how such mechanical stimulation would effect vascular network formation or an established vascular network. Most of what is known about EC response to mechanical stimuli is derived from 2D experiments to mimic the monolayer in a large blood vessel. ECs respond to stretching by forming stress fibres perpendicular to the principle strain axis [173], signalling to neighbouring cells via calcium [174], and increasing proliferation [175]. However cyclical stretching of ECs also results in a loss of barrier integrity [176].

2.4.2 Fluid Shear

Fluid shear is believed to be the principal means by which bone cells sense their environment. Osteocytes reside in a network in bone, connected by their cellular processes that traverse the bone tissue through canaliculi. External forces are

transduced through fluid shear in these canaliculi which is sensed by the osteocyte, which in turn expresses factors to control bone formation, resorption, and angiogenesis [177]. Displacement of fluids due to compression, or interstitial flow imposed by pressure differentials will impart a shear stress on cells, thus shear is a key consideration for EC network formation in mechanically loaded applications.

In the context of network formation, a number of studies have demonstrated that endothelial cells show reduced sprouting from engineered lumen when exposed to shear stress [178-180]. However, there is also evidence that low levels of shear stress (3 dyn/cm^2) can actually improve network length [181], and even higher levels of shear stress (15 dyn/cm^2) have also been reported to improve network formation [182]. Its difficult to understand the discrepancies in these results, however the change in nutrient and waste transport may at least partially account for them. In vivo, the shear stress experienced by recently anastomosed vessels induces vessel maturation; supporting a role for shear stress being inhibitory of vessel sprouting [182]. Thus, care must be taken not to overexpose ECs to shear during network formation as it may be detrimental.

Similarly, once vessels are formed, flow through the network imparts shear on the luminal side of the vessel walls. Shear stress in these situations is typically determined theoretically using the Poiseuille equation $\tau = 4Q\mu/\pi r^3$, where Q is the volume flow rate, μ is the perfusate viscosity, and r is the radius of the lumen. The heterogeneous vessel lengths, branches and diameters in a self-assembled vascular network means the imposed flow is divided up amongst the multiple vascular routes. One approach to estimate shear stress in such networks has been to assume flow in the network is analogous to flow through multiple parallel pipes [183]. Shear stresses are important in regulating vessel maturity, and thus may be critical for generating long term vascularised bone cultures [184],[185].

The driving pressure required for perfusion can also expose ECs to interstitial flow. Pressure differences between the inlet and outlet in a porous medium, such as ECM, results in interstitial flow that has been shown to be a strong modulator of vascular network formation. When simultaneously exposed to a VEGF gradient and interstitial flow, ECs underwent angiogenic sprouting towards the higher VEGF concentration, but invasion into the matrix was amplified by interstitial flow, irrespective of flow

direction [180]. Similar results were observed in a more vasculogenic context, where ECs were seeded in a bulk hydrogel [186]. This study was subsequently expanded to investigate if the ECM composition (collagen/fibrin ratio) itself accounted for any of the effects of interstitial flow [187]. It was shown that the ECM composition had a significant effect on vascularisation under interstitial flow conditions, and this effect was particular to EC subtypes; concluding that the ECM used is a critical consideration when optimising interstitial flow regimes. From these studies, it is evident that interstitial flow can have an amplifying effect on angiogenesis. Furthermore, computational analysis from these studies has calculated that interstitial flow regimes should produce very low levels of shear stress on cells (0.03-0.1 dyn/cm^2). The mechanism for the amplifying effects of interstitial flow is unknown, however, it has been hypothesised that it may help to determine the spatial distribution of MMPs and angiogenic factors as ECs degrade their matrix during angiogenesis [186].

2.4.3 Hydrostatic pressure

Hydrostatic pressure is another mode of mechanical stimulation believed to have a role in bone homeostasis, which has been incorporated into MPSs [188] and thus may be a feature of vascularised bone MPS applications. In a bone context, cyclic hydrostatic pressure induces osteogenesis in bone cell precursors [189]. The effect of this mode of mechanical stimulation is less known in ECs. Stimulation with hydrostatic pressure (6.6 kPa) has been shown to improve EC tube formation [190]. In addition, sustained hydrostatic pressure stimulated vascular EC proliferation [191]. Similarly, EC proliferation increased with increasing hydrostatic pressure between 6-18 kPa, but was shown to have a detrimental effect on VE-cadherin expression [192]. Thus, hydrostatic pressure may have a enhancing effect on EC proliferation and may indeed enhance network formation, however these effects have never been tested in a 3D context. However, consideration of barrier integrity is warranted when applying hydrostatic pressure, given its effects on VE-cadherin.

2.5 Oxygen

Oxygen tension is one of the key drivers of both vascular growth and osteogenesis. In vascular network biofabrication, the effects of oxygen tension are twofold: the direct effect of oxygen on ECs, and the indirect effect of oxygen tension on cells co-cultured

with the ECs. Directly, ECs respond to oxygen fluctuations *in vivo* by activating vasoconstrictive or vasodilative mechanisms [193] to regulate oxygen delivery. *In vitro*, experiments in 2D suggest that short term exposure to hypoxia promotes protective mechanisms [194, 195], one of which being autocrine VEGF production [196], while prolonged exposure can be detrimental [194] and inhibitive of angiogenic sprouting [197]. In addition, ANG-2, an antagonist of vessel maturation, is upregulated with hypoxia exposure [198].

2.5.1 Dissolved Oxygen

In more relevant, 3D systems, ECs are typically in co-culture, thus the direct effects of hypoxia on ECs are coupled with the indirect effects on the supporting cells. Hypoxia inducible factor 1 (HIF-1) is an oxygen-sensitive transcriptional activator that induces the transcription of a catalogue of genes involved in angiogenesis [199]. Thus, many cell types will secrete a plethora of proangiogenic factors in response to hypoxia to promote EC survival and oxygen delivery through angiogenesis. BM-MSCs, the most prominent bone cell for vascularised bone MPS applications, secrete a more potent angiogenic secretome when cultured in hypoxia compared to normoxia [200-202], and this secretome is chemotactic for ECs [203]. There is also some evidence to suggest hypoxia can rescue inhibition of network formation caused by dexamethasone [204], an essential component of osteogenic medium. The above data suggest that oxygen tension can have a large effect on both ECs and the cells that support them. However, it is unclear whether the effects are overall positive or negative.

2.5.2 Oxygen Scavenging Biomaterials

A recent paradigm in vascular network engineering involves developing gels that intrinsically produce a hypoxic environment through oxygen scavenging. While hypoxia is a key driving factor in engineering bone and other musculoskeletal tissues, it can be impractical to implement, particularly in multi organ MPS systems where different oxygen tensions are required. The Gerecht lab have produced gelatin and dextran based hydrogels with conjugated ferulic acid that consume oxygen during crosslinking, thus creating a temporary (from minutes to up to 12 hours acellular) controlled hypoxic environment within the hydrogel [205, 206]. Interestingly, these gels have been shown to have a profound effect on the vascular morphogenesis of ECs. Firstly, ECFCs incapsulated in these gels did not form vascular networks after 3 days

in thinner normoxic gels, whose thickness abrogated the oxygen scavenging effect, but formed interconnected lumenised structures in thicker gels where O_2 levels were maintained below 5% [205]. This effect was also seen *in vivo*, where hypoxia inducing hydrogels increased the density of new blood vessels surrounding the gel at 1,3 and 5 days [205]. More recently, these gels have been used to demonstrate that ECFCs, when encapsulated at high density, form stable clusters at specific regions in the gel that corresponded to 1% O_2, and subsequently sprout in an inter-cluster manner into the surrounding matrix to form connected structures [207]. While these gels have not been used in MPS systems to date, they may serve as a useful tool to induce hypoxia in a highly controllable manner in vascularised MPS systems, particularly when joining organ systems that require different oxygen tensions.

Table 1: Construction parameters for microvascular network applications

Application	ECM	Concentration	Mechanical Stim	Cell Type	Angiogenic Stim	Ref.
Vascular network construction	Fibrin	1.25-10mg/ml (Final)	N/A	HUVEC	NHLF S1P VEGF	[208]
Vascular and Neuronal Networks	Collagen	2.4 mg/ml	Applied 45 Pa hydrostatic head for perfusion	HUVEC iPSC-EC hNSC spheroids	hNSC spheroids	[55]
Breast Cancer Metastasis to Bone	Fibrin	2.5 mg/ml	0.25 dyne/cm^2	HUVEC BM-MSC	BM-MSC	[81]
Vascular Network Construction	Fibrin	2.5 mg/ml fibrinogen 0.2 mg/ml Col 1 0.15 mg/ml aprotinin	< 10 dyn/cm^2 fluid shear stress	HUVEC NHLF	NHLF	[183]
Vascular Network Construction	Fibrin	2.5 mg/ml fibrinogen	9-19 dyn/cm2 fluid shear stress	NHLF HUVECs U87MG	NHLFs	[209]
Vascular Network Construction	Fibrin + Hydroxyapatite	Fibrin – Not specified HA (0.1-0.5%)	N/A	HUVEC	NHLF HA	[147]
Vascular Network Construction	Fibrin	<10 mg / ml fibrinogen	Interstitial and Interluminal flow	NHLF ECFCS	NHLF (EGM-2MV -bFGF – VEGF)	[210]
Breast Cancer – EC Interaction	Collagen-Fibrin	2.5 mg/mL fibrinogen, 0.15 U/mL aprotinin 0.5 U/mL thrombin 0.2 mg/mL collagen	Pressure head driven flow	spheroids 4:1: NHLF:HUVEC spheroids (3) 3:1:1 NHLF:HUVEC: MCF-7	MCF-7 breast cancer cell spheroids	[211]
Vascular Network Construction	Collagen-Fibrin	Collagen 3mg/ml Gelatin 10% (w/v) Fibrin 10mg/ml	<1 dyn/cm^2 fluid shear stress	HUVECs and NHLF	NHLF	[179]
Vascular Network Construction	Fibrin	2.5mg/ml	N/A	HUVEC NHLF / BM-MSC / AD-MSC	NHLF / BM-MSC / AD-MSC	[86-88]

Application	ECM	Concentration	Mechanical Stim	Cell Type	Angiogenic Stim	Ref.
Vascular Network Construction	Fibrin	2.5-10mg/ml	N/A	HUVEC iPSC-EC	NHLF	[54]
Vascular Network Construction	Hyaluronic Acid	Not specified	N/A	hECSs hiPSCs	N/A	[52]
Vascular Network Construction	PEG + MMP + CRGDS	20 mg/ml	N/A	iPSC	Vasculife VEGF with FBS replaced with iCell ECs Medium Supplem	[144]
Vascular Network Construction	PEG + MMP + RGDS	25 mg/ml	N/A	HUVEC 10T1/2	EGM-2	[143]

3. State of the Art: Vascularised Bone Models

The primary applications of MPS systems to date has been in organs involved in drug metabolism and toxicity: intestine, lungs, liver, and kidney. Development of secondary organ models are only recently becoming more prevalent. A complete model of drug metabolism *in vitro* will require models of secondary tissues, such as bone, that are not canonically involved in these processes, but can be the target of drug side effects [212]. Currently there is no canonical state of the art vascularised bone model that can fulfil this purpose, but development is ongoing. In addition to research in drug metabolism, a vascularised bone model would serve as an ideal platform to study specific bone pathologies in which vasculature plays an integral role. As already discussed, cancer metastasis has been a popular subfield of research using these models, given that bone is a common site for secondary tumours. Additionally, many of the most common bone pathologies result from a dysregulation of bone remodelling, thus the development of models of bone remodelling is reviewed below.

3.1 Cancer

Circulating tumour cells that have disseminated from a primary tumour commonly find a site to colonise in bone tissue and form a secondary tumour; particularly in breast and prostate cancer [15]. This phenomenon has motivated the development of models of bone physiology using tissue engineering principles to try and better understand this process. Bersini et al. pre-differentiated BM-MSCs in monolayers before embedding them within a collagen matrix in an MPS as an analogue of bone tissue [82]. This model was vascularised by adding ECs to form an endothelialised channel parallel and adjacent to the bone channel. Breast cancer cells were found to extravasate through the endothelium into the bone matrix channel at higher rates compared to a control matrix, and found receptor CXCR2 and chemokine CXCL5 play a major role in this process. A similar study followed with an alternative MPS design, this time incorporating a physiologically relevant, self-assembled vascular network perfused with tumour cells. Again, this system showed that the bone mimicking environment favours metastasis, and additionally showed that adenosine is a key modulator of metastasis in skeletal tissues [81]. Hao et al. created a similar model by using an osteoblast cell line, MC3T3-E1, to create osseous tissue within an MPS

system, then seeded this with MDA-MB-231 and MDA-MB-231-BRMS (metastasis suppressed) breast cancer cells to monitor colonisation of the osseous tissue [213]. Using this model they found that metastasis suppressed cells actually colonise the osseous tissue more aggressively, but that metastasis is more frequently single cells, rather than micrometastitic cell clusters. Marturano-Kruik *et al.* also created a vascularised bone model; seeding demineralised bone matrix (DBM) with undifferentiated hBMSCs and HUVECS [214]. HUVECs self assembled a vascular networks on the DBM, which was subsequently perfused with MDA-MB-231 breast cancer cells. These cancer cells assumed a slow proliferative, drug resistant phenotype when cultured with interstitial flow. The ability to analyse and quantify metastatic adherence, extravasation and colonisation non-destructively within a bone niche in real time is a clear advantage of these systems.

3.2 Bone Remodelling

For MPS systems to augment significant advances in bone biology, creating a human *in vitro* model of bone remodelling will be essential. The coordinated actions of osteoclasts, osteoblasts and osteocytes underpin bone physiology and many of its most common pathologies, such as osteoporosis [17], Paget's disease, and renal osteodystrophy [18]. As in many processes, vasculature is critical in bone remodelling, supplying the key nutrients and factors to the bone remodelling unit during turnover. Additionally, vascular impairment is believed to be a determining factor in poor and imbalanced bone formation observed in diabetes [19]. MPS modelling of bone remodelling is in its nascency. George *et al.* fabricated remodelling on chip applications using a PDMS device that could mechanically stimulate osteocytes and provide flow conditioned media to cultures of osteoblasts and osteocytes [215]. Despite the relatively low number of published applications in bone remodelling on-chip, this field is intensely studied using conventional *in vitro* methods [8, 177]. Given the significance of vasculature in bone remodelling, MPS are an ideal platform to build more complex models of the crucial processes that contribute to the most common human skeletal conditions.

4. Current Status and Future Directions

4.1 Established Strategies for the Biofabrication of Vascularised Bone MPS

A vascularised bone MPS that supports simultaneous vascular network formation and bone matrix deposition would represent an ideal bone analogue for many applications (Figure 4.1). Such a system could indicate changes in bone anabolism as a result of novel therapies, or indicate potential adverse changes in bone vasculature, which is a hallmark of a number of bone diseases [19, 22, 216-219]. However, conditions optimal for facilitating osteogenesis can have an effect on vascular network formation; thus developing such a system is challenging [27]. This limitation has led to innovative biofabrication methods for combining *in vitro* engineered bone tissue with self-assembled vascular networks for modelling vascularised bone.

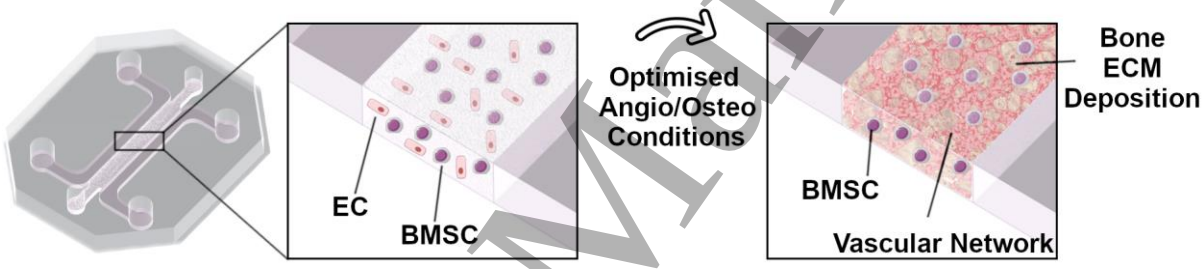

Figure 4.1: Idealised vascularised bone MPS: Simultaneously engineering vasculature and bone in an MPS as a model of vascularised bone requires the identification of culture parameters supportive of concurrent osteogenesis and vasculogenesis.

4.1.1 Pre-MPS osteogenesis

The most prominent approach to generating concurrent vascularised networks and bone tissue in MPSs has been to differentiate human bone marrow stromal cells (hBMSCs) in 2D separately before incorporating with endothelial cells and culturing to facilitate vascular network formation [81, 82] (Figure 4.2A). Once seeded in the MPS device, osteogenically differentiated hBMSCs can deposit bone related proteins such as osteocalcin and alkaline phosphatase (ALP), while also facilitating EC vasculogenesis. This approach is particularly suitable for metastatic cancer applications, for which it was designed, but may be limited in its application to other aspects of bone physiology. For example, osteogenesis and angiogenesis typically occur simultaneously *in vivo* in a process termed angiogenic-osteogenic coupling [220].

4.1.2 Decellularised Bone Matrix

Decellularised bone matrix (DBM) has been used as a scaffold to recreate a vascularised bone-like niche to study cancer cell extravasation in a MPS device [214] (Figure 4.2B). The channel in these devices contains bovine decellularised bone matrix and is seeded with hBMSCs and endothelial cells to create a bone perivascular niche. The design of the system facilitates active perfusion of the engineered bone tissue, creating a distribution of flow velocities and shear stresses as a result of the trabeculae of the DBM. This supported ECs forming capillary networks throughout the bone matrix and hBMSCs adopting a perivascular role, mimicking one of their postulated roles *in vivo* [84]. The system could be seeded with cancer cells to investigate their growth and proliferation within a bone perivascular niche.

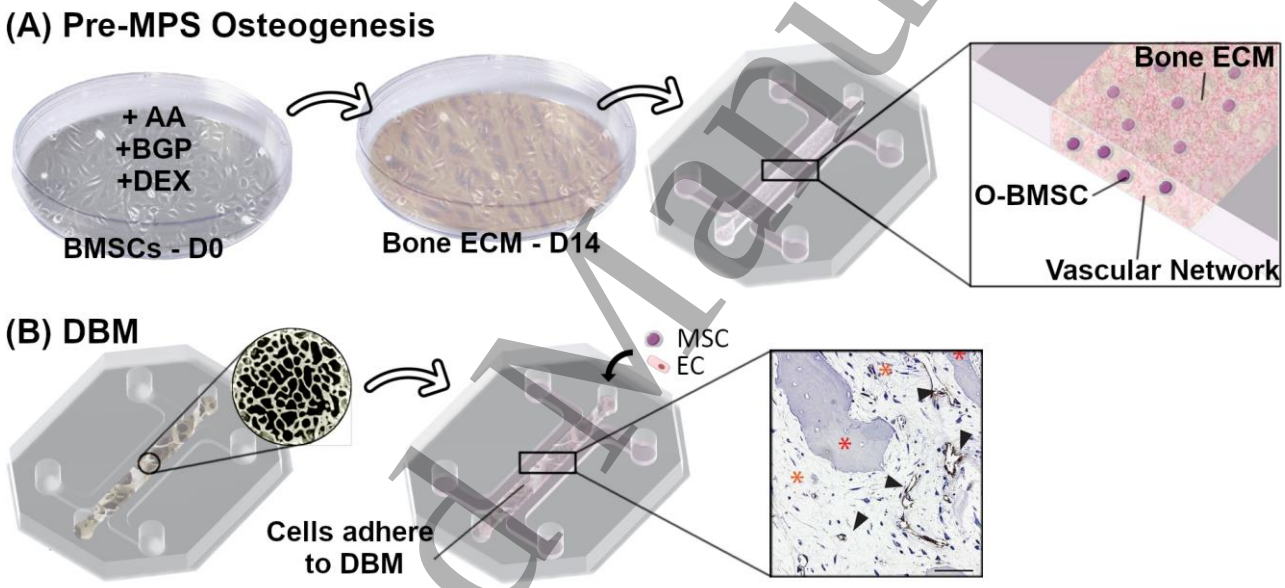

Figure 4.2: Reported biofabrication approaches for developing vascularised bone. (A) A vascularised bone tissue can be engineered by pre-differentiation hBMSCs before seeding into a MPS device. **(B)** Decellularised bone matrix can be incorporated into MPS devices for applications where bone architecture / biochemical / biophysical properties are relevant **[214].**

4.2 Prospective Vascularised Bone MPS Biofabrication Approaches

Vascularised bone MPSs have the potential to advance our understanding of bone biology and improve the efficacy of treatments for bone disease. While we have discussed the current applications of vascularised bone MPSs, the plethora of diseases and biological processes for which such systems could be applied will require

further development of existing approaches and development of new innovative approaches to recreate this complex tissue niche. A number of bone tissue engineering techniques have been developed that could be applied to developing such devices. Prospective methods for engineering vascularised bone using these techniques are shown in Figure 4.3.

4.2.1 Organoids

Organoids are self-organising aggregates of stem cells that can differentiate to become analogues of human organ systems [221]. The potential for organoids to recapitulate organ function has seen them used in a number of MPS applications such as induced pluripotent stem cell (iPSC) derived islet organoids [222], and iPSC derived human gastric organoids [223] to model pancreatic and gastric functions on chip respectively. In a bone context, the most prominent use of organoid technology is hBMSC aggregates that can form bone-like tissues; undergoing chondrogenic and subsequent hypertrophic differentiation in a process analogous to endochondral ossification that occurs in developing and regenerating bone [224, 225]. Similarly, hBMSCs can also be encapsulated in collagen microspheres to generate cartilage [226] and bone [227] *in vitro*. However, such organoids or cell microspheres have yet to be exploited to develop physiologically relevant MPS of human bone. Bone organoids or microspheres can be fabricated and differentiated separately to form a mineralised collagenous matrix and vascularised with ECs to create vascularised bone (Figure 4.3A).

4.2.2 Bone ECM

In an alternative approach, extracellular matrix (ECM) can be extracted from tissue and processed into a hydrogel for tissue engineering applications [228]. Such materials are deemed promising for recreating tissue specific environments. For example, Matrigel, a hydrogel material derived from murine tumours can recapitulate the tumour environment and thus has been useful for cancer modelling applications [229]. Similarly, bone ECM is inherently osteogenic and can drive osteogenesis of BMSCs and improves vessel infiltration in tissue engineering implants [230]. Thus, bone ECM is a promising approach to incorporate an inherently osteo-angiogenic scaffold material for vascularised bone MPS applications (Figure 4.3B). However, as

with all ECM derived scaffolds, the material is subject to donor variability, which would need to be addressed to facilitate clinical / commercial translation.

4.2.3 Controlled Osteogenic factor Delivery

Finally, the challenge of concurrently inducing osteogenesis of hBMSCs and vascular network formation of ECs may be addressed with spatially controlled osteogenesis (Figure 4.3C). Specifically, microspheres loaded with osteogenic growth factors have been developed that can release factors necessary for hBMSC osteogenesis [231]. Such an approach could be incorporated into MPS devices to locally induce osteogenesis while minimising effects vascular network formation.

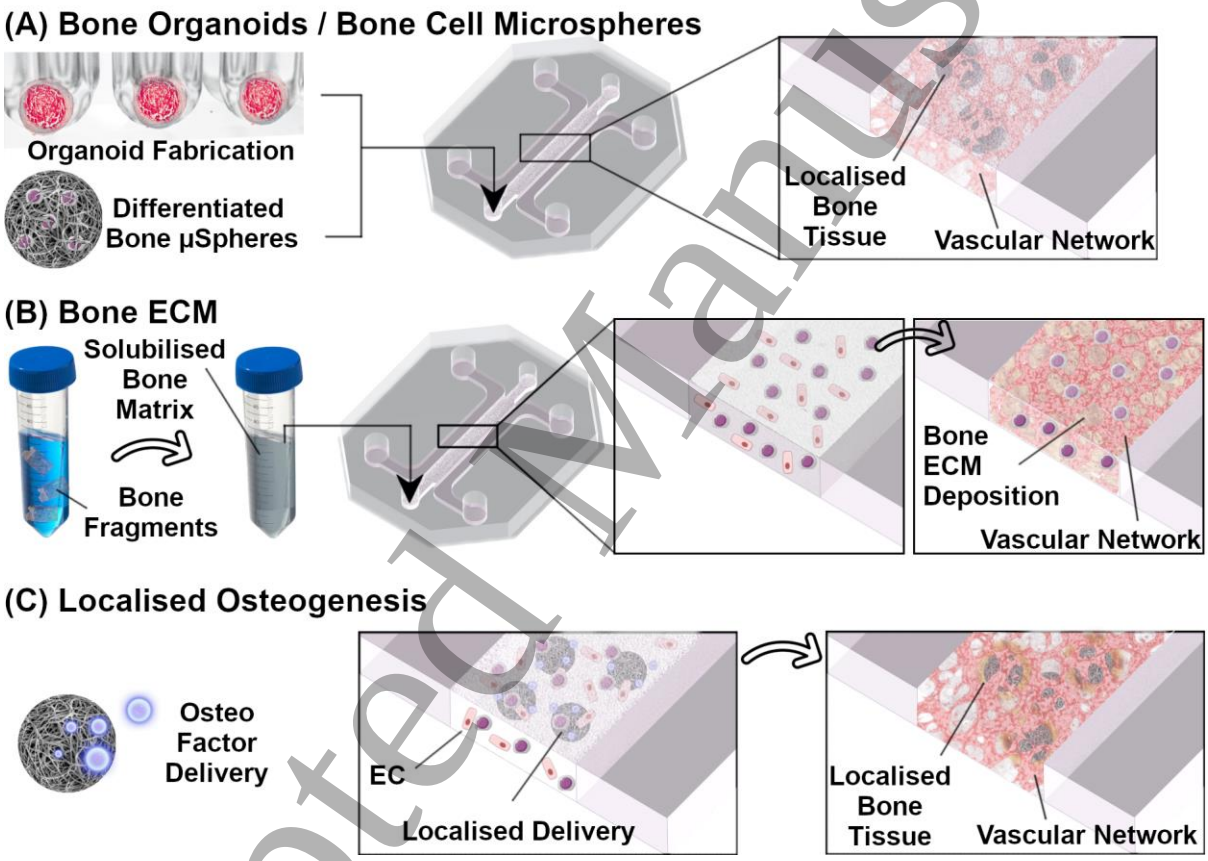

Figure 4.3 Prospective methods for vascularised bone MPS biofabrication based on current tissue engineering technologies. (A) Bone organoids and microspheres can generate discreet bone microtissues, which could be cocultured with HUVECs in a MPS as a model of vascularised bone. **(B)** The inherent cues from bone ECM could facilitate development of vascularised bone through concurrent osteogenesis and angiogenesis. **(C)** Localised osteogenic factor delivery could promote localised osteogenesis while simultaneously facilitating vascular network formation.

4.3 Analysis

With vascularised bone MPS models becoming more prevalent in the literature, a critical and arguably underappreciated area for the development of predictive bone

MPSs (and MPSs in general) is non-destructive analysis [232]. Traditional destructive analysis methods, such as PCR or immunofluorescence, are the classical methods for *in vitro* evaluation of biofabricated tissues. While these techniques are useful and necessary during application development, ideally, MPS systems should be engineered to maximise using non-destructive organ function readouts in real time. Such readouts reduce the raw materials required for experimentation, provide more information resolution in the time domain, and give instant feedback. Examples of this have been demonstrated in myoblast contraction in the neuromuscular junction [233], mitochondrial dysfunction in the liver [234], and human lung epithelium integrity using TEER [235]. In the context of vessels, ECs expressing fluorescent reporters do allow real time evaluation of network growth, and can allow processes such as extravasation [26] to be evaluated in real time. Non-destructive, functional readouts of bone will likely include non-destructive readouts of mineralisation [236], soluble indicators of bone formation and bone resorption.

4.4 MPS Product Design

Consideration of vascularised bone MPS design from the end user perspective will be essential to drive uptake of the technology. Most commonly, research MPS systems are made from PDMS, are made in batch processes limited by the number of master moulds, and require expertise to fabricate and use. Additionally PDMS, though often considered inexpensive, is indeed an expensive material compared to alternative microfluidic materials such as polycarbonate, polymethylmethacrylate, polystyrene and cyclilc olefin copolymer [237] when considering large scale production. Thus, these systems are typically far from high throughput, which is a pre-requisite for any systems aimed at drug or toxicity screening. However, companies are now bridging the gap between research and application in developing MPS technology in standard 96 and 284 well plate format, allowing screening of multiple experimental groups simultaneously [238]. These systems offer a means of studying biological processes such as angiogenesis [239], BBB function [240] and gut function [241], in a high throughput platform, that is currently being applied in pharmaceutical development [242].

In addition to throughput, material standardisation will be necessary for vascularised bone systems to become useful predictive tools. Typically, the main considerations

are the donor to donor variability that inherently comes with the use of biologically derived ECM materials, media formulations that require FBS, and the use of primary cells. Currently, there has not been any reported work on the donor variation introduced by FBS in endothelial media, or the fibrinogen / collagen materials used as ECM analogues. However, variation in primary ECs network formation and the angiogenic properties of primary bone cells is well known [243-246]. Thus, development of suitable, well characterised EC lines and bone cells for a given application will be essential for translation.

5. Conclusion

Vascularised bone MPSs also provide an ideal platform to study the role of vasculature in key bone physiological and pathological processes. In addition, such systems may provide additional insights and principles for achieving engineered vascularised bone tissue grafts for therapeutic applications, which is still major challenge for the field. Engineering vascularised bone MPSs are likely to become more prominent given their potential, thus this review stands as guidance for concurrently engineering these two critical tissue niches for modelling the bone organ. We have also outlined some of the more high level considerations to be made for developing such systems to ensure their success and translatability into commercial use.

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