Human Adipose-Derived Micro-vessel Constructs: a potential method for experimental bone regeneration

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Human Adipose-Derived Micro-vessel Constructs: a potential method for experimental bone regeneration

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Abstract

Bisphosphonates are a group of drugs widely used in medical practice to prevent bone destruction, occurring in diseases like osteoporosis and bone cancer. An unfortunate side-effect of these potent drugs is the subsequent death of areas of bone in the jaw following minor local trauma, commonly occurring during dental treatment. This unique, but well-documented, adverse effect is termed medication-related osteonecrosis of the jaws (MRONJ). While the precise cause of MRONJ is still unclear, research suggests the inhibition of new blood vessel formation, limiting the capacity for bone healing, may be a significant factor. Unfortunately, treatment options for MRONJ are very limited, and despite successful development of bone mimetic materials, most available materials do not induce sufficient formation of blood vessels. Adequate vascularisation is crucial for timely and adequate transport of nutrients and waste removal, and the provision of progenitor cells for tissue remodeling and repair.

This study provides a rationale for a new innovative approach using a human-adipose derived microvessel (HAMV)-osteoblast construct, which will potentially support local bone regeneration and healing. This project takes the important first steps in vitro, investigating the growth, migration, and viability of the HAMV within a 3D hydrogel construct and assessing the influence of HAMV development on osteogenesis, in order to provide the data necessary for subsequent testing in a pre-clinical animal model.

In this study, HAMV were seeded into tissue culture plates and cultured over 28 days, to evaluate the proliferation potential of the HAMV in a 3-dimensional (3D) collagen hydrogel construct. Growth, proliferation and morphological changes, including cell death, of HAMV

were also visualised and were examined via LIVE/DEAD® assay with the aid of confocal microscopy. The human osteoblast cell line, MG63, was cultured in conditioned media derived from the HAMV cultures to understand the influence of HAMV on bone cells. qPCR was employed to assess the changes in the relative expression of osteoblast differentiation markers during culture with HAMV conditioned media. Cell viability and morphological changes were recorded using a common cell-culture inverted microscope. Initial proliferation assay and confocal microscopy showed robust proliferation and outgrowth of HAMV from two weeks of HAMV culture. qPCR analysis demonstrated the ability of the HAMV-conditioned medium to trigger the expression of osteogenic differentiation marker/genes of bone cells by day 14, compared to cells that did not receive HAMV-conditioned media.

Overall, this study demonstrated HAMV can be incorporated into 3D collagen hydrogel and make viable deliverable constructs. This study showed that HAMV-conditioned medium has the ability to modulate the osteogenic potential of osteoblasts, suggesting that HAMV could be used as a viable tool in various regenerative medicine experiments. When considering clinical treatment aspects, this study indicates that a microvessel encapsulated in collagen hydrogel construct, derived from adipose tissue, may be an efficient therapy of MRONJ, promoting osteogenesis and vascularisation.

Statement of Originality

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

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Bridget Boston

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Abbreviations

HAMV	Human-adipose derived microvessel
VEGF	Vascular endothelial cell growth factor
BMP-2	Bone morphogenetic protein-2
OCN	Osteocalcin
ALP	Alkaline phosphatase
BMSC	Bone marrow stromal cells
VEGFR	VEGF receptor
HIF	Hypoxia-inducible factor

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I feel very fortunate to have been able to submit two articles for publishing, and have one in preparation for publishing, during the course of my degree. I could not have accomplished this without the assistance of my co-authors, particularly Dr Eugen Petcu, Dr Deepak Ipe and Dr Steven Hamlet.

ALL PAPERS INCLUDED ARE CO-AUTHORED

Acknowledgement of Papers included in this Thesis

Section 9.1 of the Griffith University Code for the Responsible Conduct of Research ("Criteria for Authorship"), in accordance with Section 5 of the Australian Code for the Responsible Conduct of Research, states:

To be named as an author, a researcher must have made a substantial scholarly contribution to the creative or scholarly work that constitutes the research output and be able to take public responsibility for at least that part of the work they contributed. Attribution of authorship depends to some extent on the discipline and publisher policies, but in all cases, authorship must be based on substantial contributions in a combination of one or more of:

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- Acknowledge all those who have contributed to the research, facilities or materials but who do not qualify as authors, such as research assistants, technical staff, and advisors on cultural or community knowledge. Obtain written consent to name individuals.

Included in this thesis are papers in *Chapters 2a/2b, 2c, and 3* which are co-authored with other researchers. My contribution to each co-authored paper is outlined at the front of the relevant chapter. The bibliographic details for these papers including <u>all</u> authors, are:

Chapter 2 a/b: Submitted to Vascular Cell (IF: 3.8)
Bridget Boston, Deepak Ipe, Bogdan Capitanescu, Stephen Hamlet, Robert Love, Iulian Nusem, Rodica Ileana Miroiu, Patrick Hans-Heinrich Warnke, Eugen Bogdan Petcu. Angiogenesis-osteogenesis coupling: a key element in bone physiology and regeneration

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Countersigned [redacted] 16/10/21

Supervisor: Dr. Deepak Ipe

Chapter One – General Introduction

As a living tissue, bone is highly vascularised and remodelled throughout life to remain intact. It consists of several different cell types: osteoblasts, osteocytes, and osteoclasts.[1] Osteoclasts, formed by the fusion of mononuclear progenitors of the macrophage-monocyte cell, permeate the interior of the bone promoting bone resorption, while in parallel, maintaining the blood calcium homeostasis.[1,2] Conversely, osteoblasts derived from local mesenchymal cells, reside along the bone surface, actively synthesising bone. Osteocytes represent inactive resting osteoblasts.[1,3] All these cells have a specific functional role in the context of intramembranous and endochondral ossification, complex processes which require the participation of several types of cells and growth factors.

Intramembranous ossification involves the direct differentiation of mesenchymal stem cells into osteoblasts and is the main mechanism leading to development of flat bones, such as the clavicle. On the other hand, endochondral ossification involves mesenchymal tissue transforming into a cartilage intermediate, which is later replaced by bone, and is typical for long bones such as the femur or tibia.[3] Bone formation also persists throughout life to alter the geometry of bones, so that they are sufficient to withstand changes in mechanical loading (modelling), to replace aged or damaged bone (remodelling) or to fill-in fractures.[57] In both modes of bone formation and at all stages of life, skeletal vasculature plays a significant role supplying not only nutritive and growth factors, but also progenitor stem cells, creating a dynamic link between the hematopoietic microenvironment, bone marrow and bone tissue. At the present time, it is known that these progenitor stem cells have a role of paramount importance, providing an adequate number of elements which differentiate into bone, cartilaginous or adipose cells. This is observed during bone growth and development, as well as during bone sculpting and remodelling.[44]

Bone marrow stromal cells (BMSC), which differentiate into cartilage, adipose tissue and stroma, modulate the formation of osteoclasts.[43] They also maintain a functionally active hematopoietic microenvironment, having a significant role in keeping an optimal pool of hematopoietic stem cells.[44] BMSC located in proximity of sinusoids express on their surface stromal derived factor 1 (SDF1) and stem cell factor (SCF), which have a significant role attracting progenitor cells in the bone marrow area.[48,49] However, a selective group of these bone marrow cells, also expressing leptin receptor (LepR), further differentiate into osteoblasts, as well as bone marrow adipose cells, through activation of the canonical Wnt molecular pathway. This is observed in both trabecular and cortical bones, both in normal physiological conditions and in bone healing and regeneration.[50,51] Recent studies have also indicated that stromal elements, which express stromal derived factor 1 (SDF1) on their surface, are also able to further differentiate into skeletal stem cells after bone trauma.[45]

Progenitor stem cells which subsequently differentiate into specialised bone cells, are derived from mesodermal cells seen in bone-marrow, more specifically the MCAM/CD146 positive subendothelial cells of the bone-marrow stroma. Subsequently, these cells can be transported via the bone marrow sinusoids into the vascular system where they can reach the bone system.[42,45] At this stage they will express a special immunophenotype characterised by CD51+CD200+CD90−CD105 or CD73.[46,47] A significant source of skeletal progenitor cells is also represented by perichondrium, which develops initially in well vascularised mesenchyme, and subsequently in the postnatal stage, providing a source of progenitor cells, which can be transferred to the growth plate and articular cartilage.[52,53]

All this data suggests that bone physiology, as well as bone repair, healing, and regeneration, depends on bone vascularisation and local angiogenesis. Frequently, these aspects have been overlooked in experimental studies evaluating various methodologies for bone regeneration, including 3D additive manufacturing. More specifically, a significant proportion of large grafts fail due to inner graft necrosis and/or a lack of integration within host tissue. Cells in the centre of large 3D tissue constructs are crucially dependent on a vascular supply of their own, as oxygen and nutrient requirements, as well as supply of skeletal stem cells, cannot be met by diffusion processes from the host tissue.[54]

A common strategy to improve bone-like construction and promote vascularisation is the stimulation of the angiogenic host tissue response at the implantation site by incorporation of angiogenic growth factors into the implants. However, the pivotal problem in vascularising implanted tissue constructs through angiogenesis is the fact that the development of new blood vessels is a time-consuming process, which can only be accelerated to a limited extent. Studies estimate that the physiological growth rate of microvessels is approximately slower than 5 μm/h. Accordingly, even highly successful proangiogenic strategies are not able to prevent cell death in the centre of large 3D tissue constructs in the initial days after implantation.[55]

Therefore, considering the requirements for healing and regeneration, the physiology of intramembranous and endochondral ossification, including an optimal supply of progenitor cells, new pre-vascularisation approaches have been developed in the field of tissue engineering. The basic idea of these approaches is the generation of preformed microvascular networks within tissue constructs prior to their implantation. In this case, the networks simply inosculate with the blood vessels of the host tissue at the implantation site. This has the major advantage of the implants being fully blood-perfused within a short period of time, independent of their size and 3D design. Unfortunately, current bone tissue engineering efforts have not been developed in parallel with the latest innovations in vascular tissue engineering. This disjunction between bone and vascular tissue engineering thus overlooks the critical inherent importance of well-developed vascular beds in the skeletal system.

Despite significant advances in biomaterials addressing many of these issues, few fabricated bone-graft constructs have found their way into clinical use. Limited success has been seen with clinical implementation of bone tissue engineering approaches, because of the challenge of reproducibly achieving hierarchical integration of the graft components from cell-cell interactions to macroscale incorporation into the host tissue. Similarly, vasculature repair and integration must be achieved at multiple levels: larger perusable blood vessel grafts are needed for restoration of blood flow into the site of injury, while smaller microvascular beds are needed to provide thorough distribution of blood across the entire scaffold volume, sustaining osteogenesis until bone function is restored.

As nutrient and waste exchange between individual cells and capillary vessels in bone is limited to small distances (100 to 300µm), designing grafts around this diffusion limit is vital for graft success. Hence, vascularisation is arguably the greatest challenge in tissue engineering and regenerative medicine, including bone tissue engineering.[56]

Aim of the study and Research Questions

This thesis will review the current data describing the interaction between vascularisation and bone remodelling and healing, while providing the rationale for a new innovative approach using a human-adipose derived microvessel (HAMV)-osteoblast construct.

The specific aims undertaken in this project are as follows:

1. To investigate the growth, migration, and viability of the HAMV in a 3D hydrogel construct.
2. Quantitate gene expression of osteoblast differentiation markers during culture with HAMV conditioned media, including the influence of HAMV on osteoblast mineralisation.

The questions which guide this study are as follows:

1. Would it be possible to develop a viable HAMV-Hydrogel 3D collagen construct which could be used to counteract bone resorption such as in MRONJ?
2. Are human adipose derived micro-vessels (HAMV) able to promote osteoblast cell growth and development?

Chapter Two - Angiogenesis-osteogenesis coupling: a key element in bone physiology and regeneration

STATEMENT OF CONTRIBUTION TO CO-AUTHORED PUBLISHED PAPER

This chapter includes a co-authored paper. The bibliographic details and status of the co-authored papers, including all authors, are:

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My contribution to the paper involved:

Providing direction on the scope and structure of the analysis, performing the search, assessing and screening the articles, preliminary analysis, and categorisation of the data into a usable format, drafting and editing the paper.

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Submitted to Aging and Disease (IF: 6.7)

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Providing direction on the scope and structure of the analysis, performing the search, assessing and screening the articles, preliminary analysis, and categorisation of the data into a usable format, drafting and editing the paper.

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Corresponding author of paper: Dr. Eugen Petcu

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Supervisor: Dr. Deepak Ipe

a. General principles of angiogenesis

A well developed and functional vascular network is of paramount importance for optimal embryogenesis, tissue healing, regeneration and reproduction. The vascular network provides oxygen and nutrients to all tissue structures, while also maintaining overall fluid homeostasis. Assembly of these vascular networks involves sprouting, migration and proliferation of endothelial cells. In the embryonic stage, we can distinguish two different types of processes that lead to formation of embryonic blood vessels, vasculogenesis and angiogenesis. Vasculogenesis can be defined as the formation of primitive vascular plexuses and the establishment of yolk sac circulation during embryogenesis, via the differentiation of endothelial precursor cells.[58,59] To achieve full potential functionality, these newly formed vascular structures require remodelling, which is achieved through angiogenesis, a complex process which includes interaction of numerous cells, growth factors and cytokines.

Angiogenesis is realised via two different mechanisms: endothelial cell sprouting and intussusceptive microvascular growth. Endothelial sprouting is made possible by endothelial cell migration, proliferation, and tube formation, whereas intussusceptive microvascular growth represents the appearance of tissue folds and columns, ultimately separating the previously formed vascular lumens, as well as formation of new vessels by in situ loop formation in the wall of veins.[59]

In early embryogenesis, the first endothelial forming elements, hemangioblasts, differentiation is modulated by fibroblast growth factors with initial formation of blood islands of the yolk sac.[58] Research conducted in patients with single-cell bone marrow transplant indicated the presence of adult hemangioblasts, suggesting that angiogenesis repair is also present in the adult population.[60] Both types of hemangioblasts seen in the bone marrow and other non-hematopoietic sites express the same immunohistochemical profile, such as CD34 and ACC133. The most important modulator for these two types of hemangioblasts is represented by vascular endothelial cell growth factor (VEGF) and its receptor, tyrosine kinase flk-1, which activate a paracrine system stimulating angiogenesis.[58]

Schatteman et al[60] suggest that hemangioblasts represent an important source of endothelial cells for physiological angiogenesis, as well as for numerous pathological conditions, being a major player in tissue repair. Another source of progenitors for endothelial differentiation is represented by circulating monocytes, which are activated by hemangioblasts.[60] The endothelial cells expand over the vascular lining, interfering with fundamental physiological and pathophysiological processes, such as vascularisation. In normal conditions, after stroke, post cancer and in homeostasis, vascular exchanges within interstitial space, fibrinolysis, and coagulation, maintain optimal vascular tonus under changing requirements, as well as cell migration and activation.[58]

The most important modulator of physiological and pathological angiogenesis, especially during embryogenesis and skeletal growth and development, is VEGF. VEGF stimulates cell division and migration, while also facilitating vascular wall permeability.[66,74] VEGF has a significant role in numerous musculoskeletal diseases, for example in promoting fracture healing, or promoting rheumatoid arthritis pathogenesis.[73] However, several molecules with similar action to the initial VEGF discovered have been found, and at present are described as the VEGF family group, for example VEGF-A, VEGF-B, VEGF-C, VEGF-D, viral VEGF-E, VEGF-F, as well as placenta growth factor (PIGF) and endocrine-derived vascular endothelial growth factor (EG-VEGF).[74] Matsumoto and Ema[78] have reported that the VEGF-A pathway is the most important during in-utero development, modulating most of the endothelial cell functions including migration, growth, and vascular wall permeability. VEGF-A is also the most important pro-angiogenic factor in the postnatal period, modulating the release of numerous paracrine factors by endothelium, subsequently improving the rate of regeneration.[78] Also, studies conducted in a mice model have indicated that VEGF-A in mice promotes robust formation of several vascular structures, such as mother vessels, glomeruloid bodies and daughter capillaries.[79]

VEGF exerts its action via several types of tyrosine kinase receptors, such as VEGFR-1, also known as Fms-like tyrosine kinase 1 (Flt-1), which is a receptor for VEGF-A, VEGF-B and PIGF, and expressed by both endothelial cells and some cancer cells.[75] VEGFR-2, also known as kinase insert domain receptor (KDR), or fetal liver kinase 1 (Flk-1), is the most important receptor, mediating the actions of VEGF when it promotes endothelial progenitor cell differentiation. VEGFR-2 also modulates pathological angiogenesis in cancer, suggesting that it may be an important therapeutic target.[76] However, its synthesis is modulated by another VEGF receptor, called VEGFR-3, or Fms-like tyrosine kinase 4 (Flt-4). This factor modulates lymphangiogenesis subsequent to VEGF-C and BEGF-D binding. However, it also promotes spouting of newly formed capillaries and overall vascular network development. Research conducted in mice with VEGFR-3 gene deletion has shown that missing this receptor results in endothelial leakage.[77] Furthermore, an overexpression of VEGFR-2 associated with a reduced amount of the cellular tightening molecule endothelial cadherin at the cell-cell junctions in postnatal animals, as well as in endothelial cultures, was found. This suggests that normally VEGFR-3 inhibits VEGFR-2 synthesis and VEGF/VEGFR2, preventing vascular leakage.[77]

Recent experimental studies conducted in a murine model indicate that fibroblast growth factor (FGF) exerts its pro-angiogenic role through c-myc regulation, which subsequently modulates production of the glycolytic enzyme, hexokinase 2 (HK2). Ultimately, an optimal level of HK2 sustains endothelial cells migration and proliferation, suggesting that FGF-dependent endothelial glycolysis is a process of paramount importance in angiogenesis.[67]

In addition, numerous authors have reported that endothelial cells differentiation and angiogenesis could be stimulated by mechanical pressure, remodelling and numerous other factors and molecules, such as interleukin-8 (IL-8), tumour necrosis factor-alpha, transforming growth factor-alpha (TGF-alpha), transforming growth factor-beta (TGF-beta), hepatocyte growth factor/scatter factor (HGF/SF), platelet endothelial cell adhesion molecule (PECAM-31), Vascular Endothelial-cadherin and ETS-transcription domain factor.[58,61,62]

Experimental research has also indicated that a new type of tyrosine kinase may interfere with angiogenesis. Apart from being present on endothelial cells, Tie-1 tyrosine kinase and Tie-2 tyrosine kinase are expressed on a certain subtype of macrophages implicated in angiogenesis and are of paramount importance to blood vessels maturation.[71] Sato et al[63] have demonstrated in a murine model that subjects deficient in Tie-1 have leaking vascular endothelium, ultimately haemorrhaging, while Tie-2 interferes directly with angiogenesis.

Tie-2 exert its action through binding to Angiopoietin 1-4 (Ang-1-4). More specifically, Ang-1 promotes Tie-2 phosphorylation, followed by an improvement of the endothelial cells' barrier.[64] No ligand has yet been identified for Tie-1, which can interfere with Tie-2 pathway after binding Tie-2. Consequently, Ang-1 is a critical player in the process of vessel maturation, mediating migration, adhesion, and survival of endothelial cells, ultimately promoting the creation of robust vessels. Additionally, it has a protective role in several important pathological conditions, including diabetic neovascularisation, diabetic nephropathy, oxygen induced-retinopathy, sepsis, and cardiac allograft transplantations.[65] Ang-2 has an interesting action. By itself it induces apoptosis and vascular regression, but it also has a synergic effect with VEGF, increasing new blood formation.[71] Remarkably, the hypoxia-inducible factor 1 (HIF-1) is a very important modulator of angiogenesis, promoting angiogenesis synergistically along with VEGF, placental growth factor (PIGF), and angiopoietins pathway.[72]

Apart from all these molecular factors, morphological components of the blood vessels are very important for functionality and development of a vascular network. It is known that the lumen of the blood vessels is lined by endothelial cells. Recent studies have suggested that endothelial cells are in fact coordinating centres for regeneration and healing processes, preventing disease. They are also part of stem cells niches and have a role of paramount importance in neurogenesis, haematopoiesis, and bone formation.[69] The vascular stem cells niches display numerous types of growth factors, called angiocrine factors, actively involved in all organ regeneration stages: induction, specification, and patterning. The synthesis of angiocrine factors is increased after any type of tissue damage, but the molecular factors involved in synthesis and release of these angiocrine molecules in time and space, are not completely elucidated.[70]

The outer surface of vessels is covered by perivascular-mural cells called pericytes, or vascular smooth muscle cells. The ontogeny of these cells is poorly understood, however it is known that if the number of pericytes is not adequate, the vessels will become dilated and haemorrhagic, causing patients to develop edema and diabetic retinopathy.[80]

It is known that development of an optimal vascular network requires fully functional endothelial cells with appropriate mobility, proliferation, and cell differentiation. However, the intricate mechanisms of vessel assembly are poorly understood. The mechanism by which endothelial cell sprouting is stopped is also not known, as well as the specific pathways and molecules involved in modulating vascular tube formation, although it is believed that all of these may be modulated by VEGF.[68]

b. General principles of bone angiogenesis

Bone, like any other tissue, requires vascularisation. Endothelium is an integral part of bone tissue, expressing a physiological paracrine function via growth factor and chemokine release, and interacting with several cellular lines. The main secretory role in angiogenesis is allocated to endothelial cells. Bone vasculature is essential for many processes, such as skeletal development and growth, bone remodelling, and healing processes. During bone remodelling, as osteoclasts move forward and reabsorb dead or damaged bone, blood vessels follow. Blood vessels also serve as a structural template for bone development and bring the key elements for bone homeostasis into the osteogenic microenvironment, such as minerals, growth factors and osteogenic progenitor cells (Figure 1).[3]

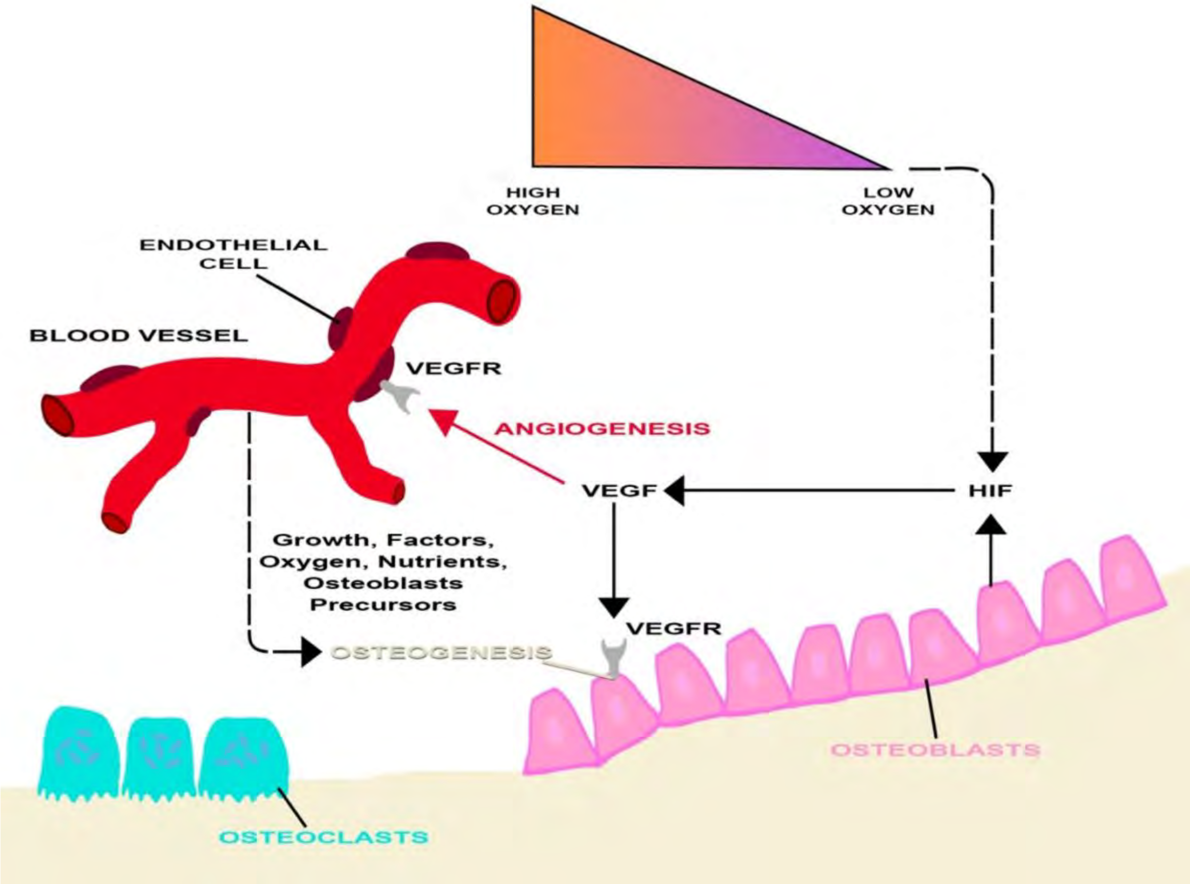


Figure 1. Angiogenesis-osteogenesis coupling

The hypoxic signalling pathway plays an essential role in coordinating osteogenesis and angiogenesis both in bone development and in postnatal bone homeostasis. Hypoxic gradients are established and promote the formation of new blood vessels which bring key elements for bone homeostasis into the osteogenic microenvironment. Replacement of the cartilaginous matrix by bone occurs through the actions of osteoblasts and

A study by Maes et al[5] confirmed the strong relationship between angiogenesis and osteogenesis, indicating that entry of osteoblast precursors into developing bones is intimately related to invasion by blood vessels. More specifically, the authors revealed that precursors exhibit a perivascular localisation and directly associate with invading blood vessels, ultimately suggesting the possibility of a coupled movement process involving mechanisms that are specific to early cells in the osteoblast lineage.[5]

In the process of endochondral ossification, the origin of trabecular osteoblasts is not known, but numerous osteoblasts are seen in the area next to the avascular cartilage. Studies conducted in transgenic mice have suggested that osterix-expressing osteoblasts precursors located in the perichondrium, differentiate into trabecular osteoblasts, as well as osteocytes and bone stromal cells. This process has been described as a typical translocation. However, some of the osteoblast precursors have also been identified in very close proximity to invading blood vessels, displaying a pericyte-like topography.[5] Remarkably, a similar pattern has been recorded in bone fractures, but in this case the outer mature osteoblasts do not show perivascular topography, the cells remain in the cortex of developing bones.[5]

Endochondral ossification and angiogenesis

Endochondral ossification is described in long bones. The process represents the transformation of embryonic cartilage into fully functional bone. In the early stages chondrocytes will proliferate and undergo hypertrophy, ultimately becoming apoptotic and dying. However, the chondrocyte also manufactures extracellular matrix which is penetrated by blood capillaries which supply the structure with nutritive elements, as well as growth factors. In addition, monocyte-macrophages and osteoclasts appear as the osteoblasts start manufacturing and laying down bone on the cartilage.[82]

Endochondral ossification is facilitated and enhanced by endochondral angiogenesis. However, endochondral angiogenesis is initiated and amplified by a group of chondrocytes located in the primary ossification centre area. These cells synthesise pro-angiogenic molecules, which promote local formation of new blood capillaries. In addition, the local osteoprogenitor cells also secrete pro-angiogenic factors, enhancing angiogenesis. These newly formed blood vessels create an extensive vascular network surrounding the chondrogenic cells. Interestingly, as the vascular network develops, ossification becomes more obvious.[82] Ossification and vessel growth develops along a longitudinal axis. In long bones, the hypertrophic chondrocytes located in the growth plates promote development of a diaphyseal and metaphyseal capillary network, which expands near the epiphyseal chondrocytes located at the extremity of the long bones. This process determines formation of the secondary ossification centres, which complete the ossification (Figure 2).[6]

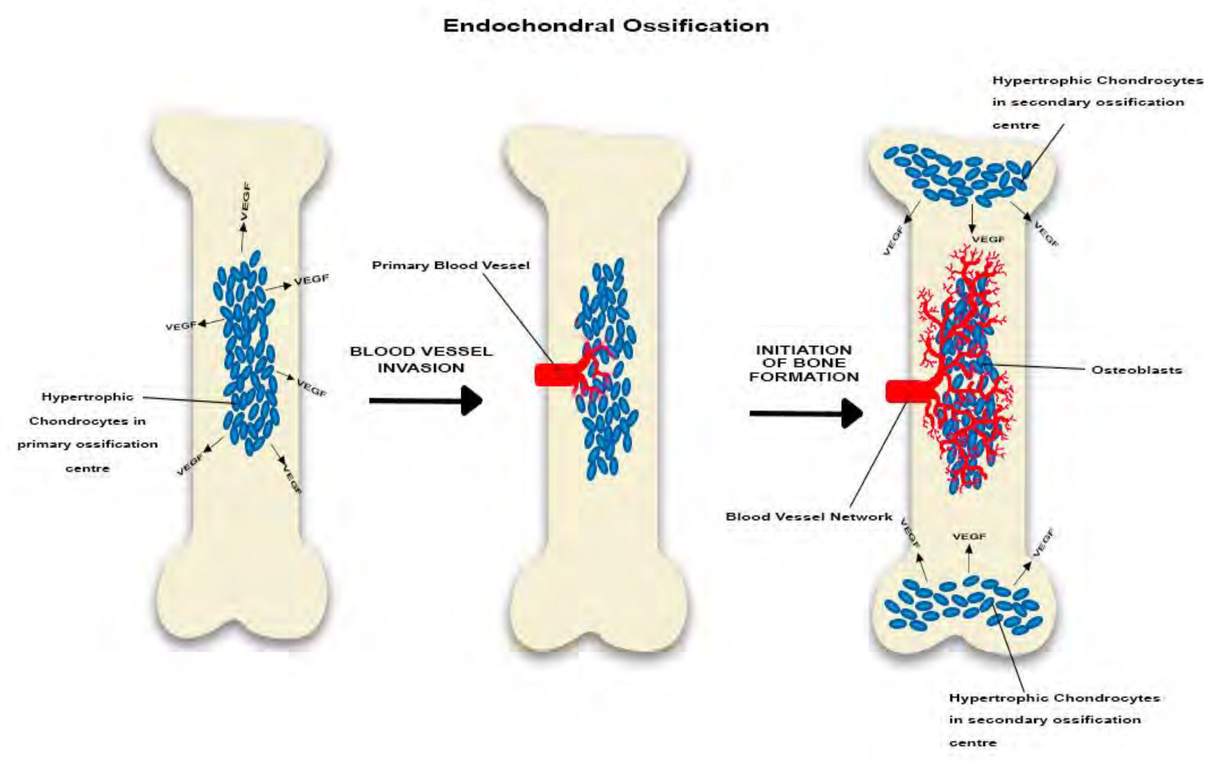


Figure 2. Endochondral ossification and angiogenesis

Hypertrophic chondrocytes located in the primary ossification centre secrete pro-angiogenic factors (VEGF) that encourage blood vessel invasion. Blood vessels extend throughout the growing bone towards the epiphysis in both directions, contributing to the formation of the secondary ossification centre. Bone growth and vascularisation advance until major structures have been established.

The action of chondrocytes is modulated by systemic factors such as thyroid factors, growth hormones (GH) and locally released factors: fibroblast growth factor (FGF), parathyroid hormone-related peptide (PTH-rP), and Indian hedgehog factor.[82] The process of endochondral ossification is modulated by the Wnt/beta-catenin molecular pathway, which promotes optimal development, inhibiting chondrocyte hypertrophy in the early stages of biological development, and later enhancing cellular hypertrophy as well as ossification. This suggests that this molecular pathway is of paramount importance for growth plate development and for cartilage boundary definition and endochondral ossification.[81] In addition, some authors have reported that chondrocyte function is also modulated by extracellular factors such as MEF2C, Runx2 and Sox 9, which interfere directly with chondrocyte gene expression. However, the action of these factors takes place in concert with expansion of the vascular network.[82]

Filipowska et al[3] suggest that specialised endothelial cells which line the blood vessels found in bone, namely type H and L, are permeable to growth factors, stimulating differentiation of bone precursor cells involved in bone regeneration. The study revealed that the bone precursor cells interact mostly with the type H endothelial cells.[3]

As the number of type H endothelial cells is age-dependent, a gradual decrease over time leads to age-related loss of bone precursor cells, explaining the diminished regeneration potential of the bone observed in the elderly.[3] Research conducted by other groups confirm that activation of type H endothelial cells promote osteogenesis.[6,7] Kusumbe et al[6] have discovered that although new blood vessel formation and osteogenesis are linked, a new type of capillary would produce specific molecules, which would facilitate angiogenesis-osteogenesis coupling, as well as maintaining an optimal amount of osteoprogenitor cells, in a murine model.[6] The most important element, type H endothelial cells, modulates the functionality of the local blood vessels, while providing a niche for the osteoprogenitor cells. The development of H type blood vessels is enhanced by HIF and Notch molecular pathways, as well as estrogen. The decline in the HIF and Notch pathways, as well as estrogen levels, is associated with a decrease in type H capillaries and osteoprogenitor cells, resulting in impairment of bone formation and osteoporosis.[6]

Xie et al[84] have described another type of blood vessels which facilitates the angiogenesis-osteogenesis coupling. These vessels are positive for CD31 and endomucin. However, studies conducted in a murine model have demonstrated that platelet-derived growth factor-BB (PDGF-BB) depleted mice have significantly low trabecular and cortical bone mass, as well as less CD31-endomucin positive blood vessels, compared with the normal subjects. Administration of PDGF-BB increases the number of osteoclasts, but also increases the number of CD31-endomucin capillaries, promoting bone formation.[84] Recent research suggests that CD31-endomucin blood vessels are also modulated by the G protein-coupled receptor kinase 2 interacting protein-1 (GIT1). This is a scaffold protein of paramount importance in bone healing and remodelling, promoting angiogenesis-osteogenesis coupling.[85] Studies performed in GIT1 knock out mice show a significant decrease in trabecular bone mass and CD31-endomucin blood vessels. In all these subjects the concentration of PDGF-BB was significantly decreased.[85]

Additionally, recent experimental studies conducted in a murine model suggest that CD31-endomucin blood vessels are also positive for miR-497∼195 cluster. A decrease in this marker is associated with lower bone mass. However, in transgenic subjects, characterised by an increase in miR-497∼195 in the endothelial cells, there is less bone loss, as this cluster facilitates HIF and Notch functionality. In elderly subjects, intravenous injection of aptameragomiR-195 promotes bone formation and CD31endomucin blood vessels.[86] This could represent a better therapeutic approach for those with osteoporosis.

Langen et al[83] indicate that the endothelial vasculature of the postnatal long bones includes a special type of endothelial cells named E. These cells sustain the osteoblasts. If the relevant endothelial integrin β1 is abnormal or is missing, the subject shows both deficient bone growth and deficient endothelial/vascular differentiation.[83]

Intramembranous ossification and angiogenesis

Intramembranous ossification is described in flat bones and includes several events characterised by expression of chondrogenic and osteogenic markers, including bone morphogenic proteins (BMPs), which modulate transformation of neural crest-derived mesenchyme to osteogenesis.[87]

Compared with endochondral ossification, this process does not require a cartilaginous stage, but robust angiogenesis is a sine qua non condition. Blood vessel formation during intramembranous ossification was first described by Thompson et al[4] who examined intramembranous bone formation in the developing frontal bone of chick embryos and demonstrated the stages of blood vessel invasion. Shortly before the initial ossification of the frontal bone, capillaries move into the thin avascular layer of loose mesenchyme which surrounds the mesenchymal condensation centre where mesenchymal cells secrete VEGF, attracting endothelial cells.[4] The capillaries then invade the mesenchymal condensation centre at or nearby the site of initial ossification.[4] Bone then undergoes mineralisation and associates with the internal and external extensive network of capillaries.[4] As bone expansion continues, a further cascade of vascular invasion and ossification continues where osteoblasts participate in mineralisation, laying down new bone at the site of large capillaries (Figure 3).

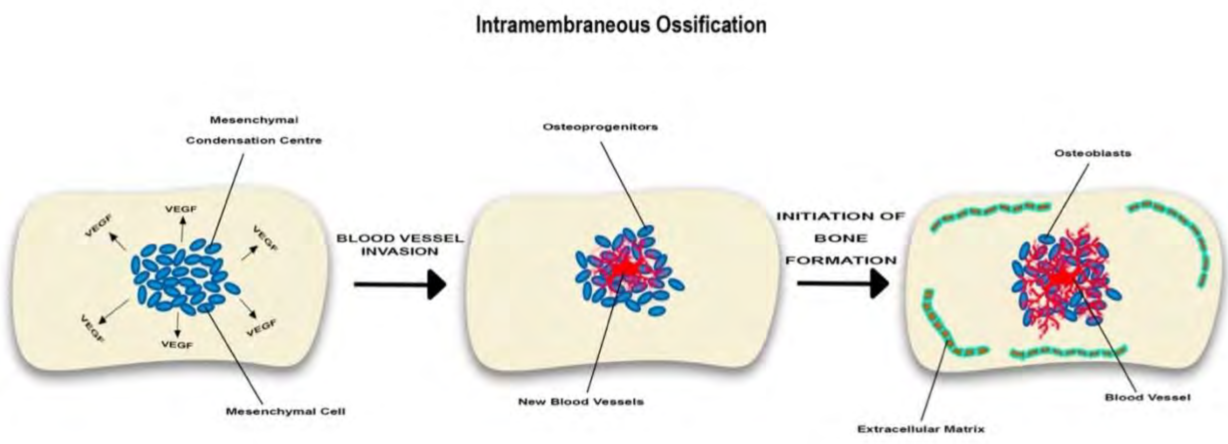


Figure 3. Intramembranous ossification and angiogenesis

Mesenchymal cells condense to form sponge-like structures and secrete pro-angiogenic factors (VEGF) that attract endothelial cells, encouraging blood vessel invasion. Mesenchymal cells then differentiate into osteoprogenitors and osteoblasts. The subsequent vascularization of the developing flat bone promotes osteogenesis. As bone expansion continues, a further cascade of vascular invasion and ossification continues.

In conclusion, angiogenesis is of paramount importance for embryonic and postnatal bone development, providing the necessary oxygen and nutrients, as well as the osteoprogenitor cells, which in turn provide the basis for continuous bone regeneration. Recent research has revealed the crucial role of new elements such as endothelial H cells, as well as HIF and Notch molecular pathways, CD31-endomucin capillaries and miR-497∼195 endothelial clusters.

Theoretically all of these could represent potential therapeutic targets. However, more experimental studies are required for validation before clinical trials.

c. Medication-related osteonecrosis of jaw (MRONJ) in elderly patients: clinical aspects and molecular pathways.

Introduction

At the present time, despite a significant increase in the average life span and numerous individuals living in countries with a high standard of living, the elderly are frequently affected by pathological problems related to their skeletal system, which ultimately impairs their mobility and quality of life. In this age group, the most important diseases associated with bone destruction are osteoporosis and malignancies with bone metastasis.

The World Health Organisation[89] indicates that osteoporosis has been diagnosed clinically and radiologically in more than 75 million people living in Europe, North American and Japan.[89] However, it is reasonable to assume that the real number of individuals suffering from osteoporosis is higher. Osteoporosis determines significant morbidity with a serious socio-economic impact. In this context, 4.5 million osteoporosis-related fractures are recorded every year in Europe, North, and South America. Overall, osteoporosis-related fractures are associated with 2.8 million disability-adjusted life years (DALYs), significantly higher than the DALYs determined by conditions such as hypertension.[89] Therefore, to counteract bone resorption induced by osteoporosis, these patients are treated with various pharmacological agents including bisphosphonates.

According to a recent study conducted by the International Agency for Research on Cancer, in 2020, more than 19 million new patients with cancer and nearly 10 million deaths due to cancer were recorded. More specifically, 2.3 million new breast cancer cases were recorded and more than 7% of all new cases were prostate cancer.[90] Both these types of cancers, as well as others such as the gastrointestinal and skin cancers, develop in their natural evolution to osteolytic bone metastasis, characterised by significant bone resorption and hypercalcemia of malignancy. However, the development of new chemotherapeutic agents combined with an early diagnosis, have made possible a cure, or at least long-term survival for numerous cancer patients. These patients often receive bisphosphonates to prevent the development of osteolytic metastases or anti-angiogenic agents to halt any further development of cancer. Sharma et al[20] indicate that bisphosphonates are administered as anti-resorptive agents in clinical settings such as cancer and osteoporosis [20], but they also have an analgesic role.[20] However, after minor dental interventions or dental trauma, patients treated with bisphosphonates develop a condition which was initially labeled bisphosphonate osteonecrosis of jaw (BRONJ). The condition could be explained by the anti-angiogenic effect induced by administration of bisphosphonates, such as pamidronate and zoledronic, in patients with hypercalcemia of malignancy or osteoporosis-induced hypercalcemia.[20,92] However, other studies have indicated that jawbone osteonecrosis follows therapy with other types of pharmacological agents, and the label 'medication-related osteonecrosis of jaw (MRONJ)' has largely replaced the BRONJ terminology. MRONJ can be described after three types of pharmacological agents: bisphosphonates, denosumab or anti-angiogenic pharmacological agents used in cancer therapy.[93] For example, Mauceri et al[14] have indicated that MRONJ is described after therapy with anti-vascular endothelial growth factor (anti-VEGF) monoclonal antibody, mammalian target of rapamycin (mTOR) inhibitors, receptor activator of nuclear factor κB ligand (RANKL) inhibitor and tyrosine kinase inhibitors (TKIs), all anti-angiogenic factors. Another example refers to non-healing osteonecrosis of the maxilla and mandible in patients treated with denosumab, a monoclonal antibody and RANKL inhibitor, used to counteract bone destruction associated with osteolytic cancer lesions.[8] MRONJ has also been associated with herceptin and pertuzumab, monoclonal antibodies administered in Her2-positive breast cancer patients.[15] Some authors have described that in rare cases, tumour necrosis factor alpha (TNF-α) inhibitors, administered in immune-mediated inflammatory diseases such as rheumatoid arthritis and inflammatory bowel disease, may be associated with MRONJ.[16,17] MRONJ has also been described after therapy with pembrolizumab, a monoclonal antibody which blocks programmed-death 1 (PD-1) on the surface of T-cells in a variety of cancers such as lung cancer.[18] Interestingly, MRONJ has also been observed in patients with acute myelodysplastic leukemia who have been treated with bemcentinib via an anti-angiogenic mechanism and by interference with their immunological profile.[96] Owosho et al[97] have also reported cases of MRONJ after ipilimumab, a monoclonal antibody administered in patients with malignant melanoma, which acts by inhibiting the immune system via CTLA-4 activation.

In this context, McGowan et al[12] have shown that in Australia the incidence of MRONJ has continuously increased since 2003. During this time, it was recorded that more than half of MRONJ cases is associated with low-dose bisphosphonates used in the treatment of osteoporosis, and 47% are related to cancer patients treated with a high-dose anti-resorptive medication. Limones et al[13] have revealed that in cancer patients, the incidence of MRONJ ranges from 2.3% after 3 years therapy with zoledronic acid, to 3.2% after 3 years treatment with denosumab. However, in patients with metastatic breast cancer followed for 77 months, the incidence of MRONJ increases to 4.1% and 13.6% after zoledronic acid and denosumab therapy respectively.[98] In addition, MRONJ was described in patients receiving therapy for Paget's disease (3.3%) and rheumatoid arthritis (4.6%).[12]

From a histological perspective, MRONJ is characterised by the presence of osteoclasts, inflammatory cells, and reactive bone formation, all in close proximity to necrosis.[99] All of these could explain the oral pain and dysphagia described in cancer patients with MRONJ.[11]

However, at the present time, therapy for MRONJ patients is not satisfactory, and the molecular factors and pathways promoting the development of the bone destruction seen in MRONJ, are poorly understood.

MRONJ clinical features and classification

A significant number of MRONJ patients (94%) present with asymptomatic exposure of bone, while up to 4.5% develop mandibular fractures after dental therapy.[8] Therefore, the early detection of MRONJ before development of bone exposure is of paramount importance. In this context, it seems that the earliest signs of MRONJ are suggested by radiological evaluation, which identifies osteolysis and the associated osteosclerosis, increased thickness of lamina dura, enlargement of the periodontal ligament space, increased thickness of the mandibular cortex, enhancement of the mandibular canal and periodontal bone destruction.[100,10]

Initially, Weitzman R[101] suggested that MRONJ lesions should be classified according to the size of necrosis and severity. The size is measured as the largest diameter of single and multiple lesions: stage 1, less than 0.5cm, stage 2, larger than 0.5cm but less than 0.99cm, stage 3, less than 2cm, and stage 4, lesion(s) larger than 2cm.[101] Based on this suggestion and the extent of clinical features, the American Association of Oral and Maxillofacial Surgeons (AAOMS) proposed a classification of MRONJ in four stages, with an additional "at risk" group describing those that have received anti-resorptive medication.[102]

Stage 0 is characterised by non-exposed bone, but abnormal radiological tests and variable pain, while stage 1, fistulas that lead to the bone as well as bone necrosis without evidence of infection, can be identified. Compared with stage 1, in stage 2 there are obvious signs of infection with erythema and purulent discharge (Figure 4; Figure 5).

Figure 4. Patient with stage 2 MRONJ (AAOMS criteria)

Photo represents an elderly edentulous patient with stage 2 MRONJ according to AAOMS criteria, but stage 1 according to Weitzman criteria as the lesion measures less than 0.5 cm.

Figure 5. Patient with stage 2 MRONJ (AAOMS criteria)

Photo was taken from an 88-year-old female patient with stage 2 MRONJ according to AAOMS criteria, but stage 4 according to Weitzman criteria as the lesion expanded over more than 2 cm.

The most severe stage, stage 3, is characterised by exposed and necrotic bone, extensive infection and one or several associated features such as: pathologic fracture, extraoral fistulas, oral antral/oral nasal communications, osteolysis involving the inferior border of the sinus, or necrosis involving the zygomatic bone, mandible, and maxillary sinus (Figure 6; Figure 7).[102]

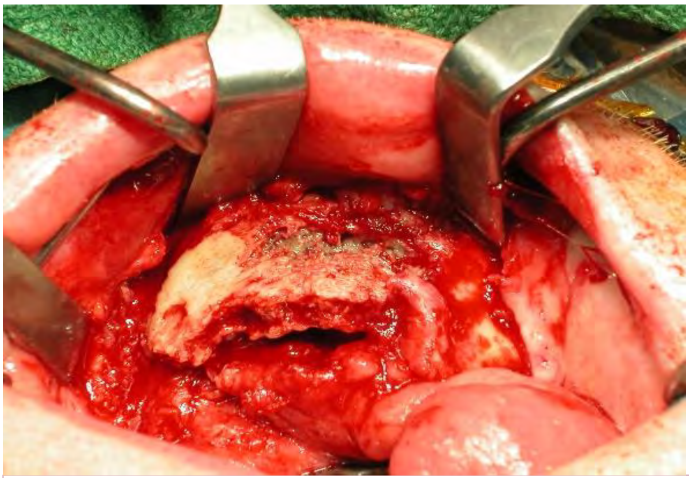


Figure 6. Patient with stage 3 MRONJ (AAOMS criteria)

72-year-old female with Stage 3 MRONJ according to AAOMS criteria treated by surgical resection. There is obvious extensive necrosis and haemorrhage.

Figure 7. MRONJ stage 3 resection specimen

Photo represents a stage 3 MRONJ resection specimen in which the necrosis was extending into the maxillary bone in a 72-year-old female patient.

Physiopathology

Recent theories suggest that MRONJ is the result of inhibition of local angiogenesis and osteoclasts activity in concert with local inflammation and/or infection.[103] Yapijakis et al[104] suggest that MRONJ might have a genetic basis, due to identification within a sub-group of hypertensive patients. More specifically, it seems that MRONJ develops in patients with hypertension that have the D variant of *ace* gene. This was described in both homozygous and heterozygous individuals for D-*ace* gene.[104] Additionally, patients with inflammatory rheumatic conditions have an increased risk if they have osteoporosis treated with bisphosphonates.[105] Therefore, it is best to assume that MRONJ has a multi-factorial pathogenesis, the toxic effect on bone cells and the anti-angiogenic mechanism of bisphosphonates being of paramount importance.[27,24,25]

Histological and molecular evaluation of tissue taken from MRONJ patients has revealed that the condition is characterised by numerous multinucleated, giant osteoclasts. A dendritic cell-specific transmembrane protein (DC-STAMP) is also noted in the presence of cell-cell fusion of osteoclasts, an element indicating active osteoclast formation. This would suggest that cell-cell fusion is a key element in the physiopathology of this condition, but the significance of these findings is poorly understood.[27]

The anti-angiogenic effect induced by both bisphosphonates and some anti-cancer pharmacological agents, inhibits endothelial cells growth and development, promoting necrosis.[106] Moreover, Ferretti et al[21] have shown that in patients with metastatic breast cancer, zoledronic acid promotes a reduction in VEGF and angiogenesis as well as FGF-2 and MMP-2, altering the tissue environment status quo.

Remarkably, zoledronate and risedronate inhibit angiogenesis, but this effect is independent of biochemical pathways leading to osteoclast dysfunction. This suggests that these two effects induced by bisphosphonates are independent of each other.[107] Interestingly, in animal models with experimental periodontitis, low dose risedronate inhibits osteoclast function and bone resorption, but higher dosage impairs angiogenesis.[23] Kaneko et al[109] have reported that zoledronic acid increases the local inflammation, promoting overexpression of IL-1 beta via an NLRP3 inflammasome. Overall, local inflammation would worsen bone destruction induced independent of this mechanism.

Decaux and Magremanne[18] indicate that MRONJ could also be the result of decreased bone turnover after alteration of osteoclasts functionality induced by chemotherapeutic agents, such as epacadostat and pembrolizumab. Also, as in the case of bisphosphonate therapy, the associated infection and inflammation accelerates the course of osteonecrosis.[18]

More recently, Isawa et al[110] have indicated that denosumab acts as an anti-RANKL antibody. It is known that RANKL, a member of the RANK-RANKL-OPG pathways, is a very important factor activating osteoclast genesis. Not only is RANKL expressed by osteoclasts but also by T lymphocytes, promoting osteoclastic cell growth and development via an immunological mechanism.[18] Since denosumab has an anti-RANKL action, it impairs osteoclast cell formation. Experimental studies conducted in a murine model have shown that subjects treated with denosumab have impaired osteoclast genesis while displaying normal tooth growth.[110] Therefore, in some cases of MRONJ described after denosumab therapy, other molecular mechanisms including an anti-angiogenic pathway must be activated. The associated infection, induced and promoted by a non-healing MRONJ lesion, is also a very important factor which needs further investigation. Recent studies have indicated that both bisphosphonates and anti-RANKL agents induce local synthesis of antimicrobial peptides (AMPs) such as human alpha and beta defensins, which could have an etiopathogenic role in MRONJ. According to Thiel et al[112] defensins might represent a therapeutic target in patients with MRONJ induced by dental trauma after anti-resorptive therapy.

However, despite all this data at the transcriptional level, the mechanisms of MRONJ have not been thoroughly investigated. Recent research suggests that several factors that modulate the functionality of osteoclasts could have a major role in the physiopathology of this condition. In this context, NFATc1, a major upstream activator of osteoclasts, and BCL6, a factor with robust anti-osteoclastic action, might have a role of paramount importance in MRONJ pathogenesis.[113] However, their role is yet to be fully understood and more experimental data is needed, as important clues may elucidate the precise mechanism and the factors involved in MRONJ physiopathology and pathogenesis.

MRONJ in patients with osteoporosis

MRONJ has been described in 2% of osteoporosis patients treated with bisphosphonates.[94] In a study conducted in 283 patients presenting with mandible necrosis, only 25.6% met the diagnostic criteria for MRONJ. However, 52.5% of these MRONJ patients were taking anti-resorptive therapy including bisphosphonates for osteoporosis.[12]

Numerous studies have reported MRONJ in association with osteoporosis after dental therapy and minor trauma. Bagan et al[114] have described MRONJ in patients treated with the anti-RANKL denosumab, but who had a history of bisphosphonates intake. In this study, the most important triggering factor was represented by the dental extraction: 90% of patients developing bone exposure, which is diagnostic for MRONJ.[114]

The data revealed by this research has been confirmed more recently by a pilot study conducted in 86 female patients with a mean age of 73.9 year (range 45-97). The study indicated that 80% of these patients were taking bisphosphonates for osteoporosis and nearly 60% developed MRONJ after tooth extraction, implant surgery and denture use.[115] A two-center retrospective study conducted in patients with bisphosphonate induced MRONJ indicated that 30% of patients developed MRONJ in association with osteoporosis, and nearly all cases (30 out 32) were triggered by dental extraction, jaw lesions being seen in more than 70% of cases. About 65% of the patients were cured by conservative and surgical therapies, while the remaining cases did not heal.[116] The authors have determined that orally administered bisphosphonates, over shorter duration of time, as well as the localisation of necrosis in the area of frontal and premolar maxilla, represent a better outcome.[116]

A recent retrospective study using the Japanese Adverse Drug Event Report database found 3875 MRONJ cases, often occurring after 70 years, in osteoporosis patients treated with either bisphosphonates or anti-RANKL denosumab. However, it seems that the most common factor promoting MRONJ is represented by zoledronic acid (56%), and most patients are women (68.1%). Unfortunately, non-healing was recorded in more than 30% of cases after zoledronic acid, and in nearly 50% of patients treated with denosumab.[117]

Although recently, these results have been contradicted by a study by Lesclous et al[118], which highlighted that women with osteoporosis treated with bisphosphonates, can safely undergo tooth extraction without development of MRONJ, if treated conservatively with chlorohexidine and antibiotics only, for local infections.[118] However, these results must be regarded with caution, and further data should be obtained from other clinical trials replicating the conditions described in this study.

Considering that anti-osteoporosis therapy is associated with MRONJ, a recent theory suggests that mild to moderate osteoporosis is a positive factor promoting bone functionality and enhancing self-regeneration. It is widely accepted that bone regeneration depends on availability of stem cells, as well as vascular supply, and a balance between osteogenic and osteoclastic factors. As people age, the number of progenitor stem cells drops, and the local vasculature needs to supply essential factors and progenitor cells to sustain normal bone self-renewal. Theoretically, this could be facilitated by an osteoporotic bone which the authors suggest could hold a larger proportion of blood vessels, providing an increased amount of stem cells and growth factors locally, ultimately sustaining robust bone development. It appears that anti-osteoporotic anti-resorptive medication increases mineralisation, reducing the area available for vasculature and subsequently reducing the supply of stem cells, pro-osteogenic, and pro-osteoclastic molecules.[119]

MRONJ in patients with cancer

Numerous studies have revealed that compared with the number of the elderly patients with osteoporosis developing MRONJ after bisphosphonates or anti-RANKL denosumab, the incidence of MRONJ is significantly higher in cancer patients receiving the same type of anti-resorptive therapy.[120] Poxleitner et al[94] indicate that MRONJ is reported after antiresorptive therapy in up to 20% of cancer patients who develop bone metastases induced by solid and hematological tumors.[94] Also, Svejda et al[95] suggest that MRONJ develops in 15% of cancer patients, as in these cases the administration of high doses of bisphosphonates, anti-RANKL and anti-angiogenic agents is required at shorter intervals of time. In such instances, the development of MRONJ seems to be accelerated by concomitant administration of glucocorticoid medication, diabetes mellitus, and any other cause that promotes a local inflammatory reaction, such as poorly fitting dentures or inappropriate oral hygiene.[95]

New trends in MRONJ therapy

There is no "gold standard therapy" for MRONJ. In this context, a detailed analysis of five randomised controlled trials conducted on 1218 patients diagnosed with MRONJ, investigating the outcomes of both prophylactic measures as well as therapeutic measures, suggest that a follow-up conducted at 3-months is very important in those with associated cancer who have been treated with intravenous bisphosphonates. The study indicated that at the present time there is no optimal intervention for MRONJ prophylaxis, and no definite conclusion can be reached regarding fluorescent guided tetracycline fluorescent surgery, and hyperbaric oxygen therapy.[108]

In patients with advanced MRONJ, surgical resection followed by vascularised bone reconstruction, such as microvascular free flaps, seem to be the best therapeutic option.[132] Recent research suggests that for the best therapeutic outcome, the therapy of MRONJ patients should be personalised, based on the pattern of periosteal reaction, which is described in more than 20% of MRONJ patients. Soutome et al[121], indicate that while surgical therapy in these patients is better than a conservative approach, the outcome depends on the presence of a periosteal reaction. MRONJ patients without such periosteal inflammation have the best clinical outcome.[121]

The type 1 periosteal reaction is characterised by new bone formation parallel with the mandible, without any interposing gap, while type 2 lesions are diagnosed by the presence of a gap between newly formed bone and the mandible. However, the most severe lesion is represented by the periosteal reaction type 3, diagnosed through the presence of a largely irregular space between the mandible and the newly formed bone.[121] Overall, it has been suggested that efficient therapy for MRONJ patients requires that any osteolytic areas, as well as any type 3 periosteal lesion, be surgically removed.

Recently, other non-surgical pharmacological anti-MRONJ agents have been tested. A clinical study conducted in 47 patients with MRONJ has revealed that administration of 20 μg/day of teriparatide, a recombinant fragment of human parathyroid hormone, for two months, is efficient in decreasing bone destruction.[122] Other authors reported that teriparatide improved MRONJ after six months of therapy.[123] However, more clinical studies are needed before the role of this agent can be established. Additionally, some authors have reported that vitamin D may prevent the development of MRONJ in elderly patients.[103] However, the precise molecular mechanism is not known.

Recently, regenerative approaches have been tried in MRONJ. However, despite the successful development of bone mimetic materials that can replace damaged bone from a structural point of view, most of the available bone biomaterials do not induce sufficient formation of blood vessels. This lack of a functional vasculature to support the graft is the biggest bottleneck for cell-based regenerative therapies. In bone tissue engineering, adequate vascularisation is crucial for timely and adequate transport of nutrients and waste removal, and the provision of progenitor cells for tissue remodeling and repair. Indeed, vascularisation and bone formation are highly linked as vascularisation precedes osteogenesis during both embryonic development and adult bone healing, which is characterised by several phases, including a proliferative phase in which angiogenesis is a major component.[124] Angiogenesis, the formation of new blood vessels via sprouting, is a complex process whereby endothelial cells migrate out from pre-existing vessels and form new connections to increase the vascular network. Moreover, the vasculature in bone appears to be formed mainly, or perhaps even exclusively, by angiogenesis. This vascularisation event in the intramembranous jaw bones occurs in a similar fashion to that seen during endochondral angiogenesis, which suggests that similar molecular mechanisms are involved.[125] Mesenchymal cells condense to form sponge-like structures and differentiate into osteoprogenitors and osteoblasts which secrete ECM and form ossification centers, and, ultimately, fully differentiated osteocytes. Matrix proteins and pro-angiogenic factors generated by the ossification centers then attract blood vessels. The subsequent vascularisation of the developing flat bone then promotes osteogenesis.[125]

In this context, it is common knowledge that poor angiogenesis is a frequent and vital barrier to tissue regeneration. Regenerating tissue over 200nm exceeds the capacity of nutrient supply and waste removal from the tissue, and therefore requires a well-developed network of blood vessels.[129] A local well developed vascular network with fully functional endothelial cells is of paramount importance in this setting. However, bisphosphonates and other anti-resorptive agents have a major inhibitory action on endothelial cells and progenitor elements, as well as on the micro-vessel sprouting. Therefore, the greatest issue in MRONJ therapy remains the counteracting of the direct anti-angiogenesis induced by a variety of pharmacological agents.[126]

Experimental research conducted in a murine model has revealed that MRONJ therapy with endothelial progenitor cells (EPCs) is very efficient, significantly decreasing necrosis while increasing VEGF levels in serum and tissue, ultimately improving fibroblast and epithelial cell functions.[130]

Moreover, recent in-vitro studies have provided encouraging data indicating that the addition of endothelial progenitors would prevent the anti-fibroblastic effect of zoledronic acid and dexamethasone, increasing vascularisation, and ultimately preventing MRONJ.[127] This has led to several types of tissue engineering approaches combining the use of angiogenic growth factors and/or transplantation of proangiogenic cells, such as endothelial progenitor cells with scaffolds. Whilst feasible, in vivo recapitulation of the events involved in appropriate cellular differentiation, proliferation and formation into functional structures, is very difficult. However, the use of only proangiogenic cells also has significant disadvantages, since perivascular cells, including mural cells, are obligatory for the formation of native, multilayered mature microvessels.[131] Therefore, proangiogenic cells should be used in parallel with vascular growth factors such as VEGF, as well as other pro-angiogenic molecules embedded in hydrogel scaffolds.

A pilot clinical study conducted in patients with stage II and III MRONJ, has recently revealed that these patients could be successfully treated using a scaffold containing l-platelet-rich fibrin (L-PRF) and adipose-tissue stromal vascular fraction (SVF), which includes endothelial progenitor cells (EPC) and mesenchymal stromal cells (MSC). This pilot study showed that complete healing of the buccal mucosa takes place within a month and robust bone formation is noted.[128] However, this study should be further replicated to include large cohorts of patients and more clinical and laboratory data is needed to verify the safety and dynamics of this therapeutic approach.

In conclusion, MRONJ develops after dental therapy in elderly patients with osteoporosis and in patients with various types of cancer and bone metastasis who have been treated with anti-resorptive agents. Currently, there is no efficient therapy for MRONJ, and although in some cases close regular follow-ups and better local hygiene can be effective, surgery with removal of the necrotic area is still suggested. In this context new regenerative approaches have been tested in experimental and pilot studies. As vascularisation and angiogenesis is of paramount importance for bone functionality, theoretically the best method should include application of pro-angiogenic and endothelial progenitor cells and pro-angiogenic growth factors including VEGF, and other factors with similar action. As the world population ages, it is likely that the number of patients with osteoporosis and bone metastasis will increase significantly. However, as large numbers of these patients are treated with bisphosphonates and other anti-resorptive agents, the number of MRONJ cases will likely increase. Therefore, finding a new improved method to treat this condition will have a significant impact, improving the quality of life of these patients.

d. Vascularisation and 3D additive manufactured constructs for bone regeneration

The current best available therapy for significant bone destruction including osteonecrosis, described in severe MRONJ, non-union bone fractures, and other bone anomalies, is represented by auto- and allografts as well as vascularised free flaps.[139,140,141] The transfer of vascularised tissue has attracted a lot of interest and was used in the past to treat severe cranial and long bone destruction. In these cases, bone reconstruction is facilitated by "transfer" of three different flap structures characterised by the presence of penetrating and non-penetrating periosteal vessels as well as nutrient vessels.[141] Unfortunately, these methods are not always successful and if the bone defect that needs to be replaced is too extensive, such as in extensive necrosis, tumours, or congenital conditions, these approaches are not very useful.

3D additive manufactured constructs-scaffolds

During the last decade, we have witnessed new approaches in skeletal regenerative medicine which focus on using vascularised synthetic scaffolds fabricated by 3D additive manufacturing, requiring the concomitant use of mesenchymal stem cells and growth factors.[138] These approaches were developed because of the need to treat large bone defects. By introducing various factors implicated in bone regeneration, an optimal environment where they interact and ultimately facilitate bone formation, is created. In this context, it was hypothesised that a natural or synthetic biomaterial might be suitable to be used as a scaffold. This material should have robust osteogenic properties allowing the bone osteoprogenitor cells to develop into bone cells such as osteoblasts, osteocytes, and osteoclasts.[139,142] In addition, this synthetic scaffold should be osteoconductive, facilitating local cellular exchanges, as well as extracellular matrix synthesis, enhancing osteogenesis. Lastly, such biosynthetic scaffold should be osteoinductive, promoting the action of various growth factors and overall, enhancing the growth and development of bone cells.[142]

Initially, natural organic polymers were tested for a potential role in bone healing, but naturally occurring products such as fibrin, silk fibroin, glycosaminoglycans and collagen have an immunogenic role, promoting local infection and inflammation.[143,144,145,146] However, these are used along with other materials and factors in the fabrication of composite materials for 3D additive manufacturing of scaffolds.

Inorganic materials such as ceramics made of hydroxyapatite, calcium sulphates, tricalcium phosphate, as well as calcium phosphate cements and glass composites have been tested as candidates for scaffold fabrication. The most common type, calcium phosphate ceramics, including hydroxyapatite, biphasic calcium phosphates, tricalcium phosphates and bio-glass, have shown some promise, but a major disadvantage is represented by the associated brittleness.[147,148.149] Alternatively, compounds such Norian SRS, a cancellous bone cement which contains calcium phosphate, have excellent resorbing properties and have been used in the surgical therapy of distal radius fractures.[150] Other research groups have tried to incorporate calcium phosphate scaffold growth factors, creating new improved products. For example, a 3D calcium phosphate cement scaffold loaded with bovine serum albumin, VEGF, and mesenchymal cells, has shown a great potential in bone regeneration experiments. The whole construct was subsequently exposed to a humid atmosphere which prevented loss of scaffold-embedded factors. Remarkably, this procedure allowed efficient cement setting and no cracking or swelling of the product was observed.[152]

However, at the present time, it is widely accepted that polymers represent the most promising materials in 3D additive manufactured scaffolds experiments for bone regeneration. Ceccarelli et al[153] suggest that synthetic polymers such as polyglycolic acid (PGA), polylactic acid (PLA), and polycaprolactone are excellent candidates for scaffolds used in bone regeneration therapy, including vertical and horizontal bone grafts, oral sinus lift and dental socket preservation. Furthermore, PGA/PLA scaffolds created by computer-aided design and manufacturing (CAD/CAM) technology allow a personalised approach in subjects with extensive craniomaxillofacial bone lesions that need replacement, ultimately promoting infiltration by bone marrow stem cells.[154] Although there is a lack of long-term follow up data regarding this approach, we can speculate that without a well-developed vascular network this type of method would not be successful long term, unless the bone defect is small.

To better manipulate the functionality of the polymers, composite copolymers such as the copolymer poly (lactic acid -co-glycolic acid) (PLGA) have been developed. PLGA has an excellent biodegradability, but unfortunately its products of degradation promote local acidification.[157] However, when combined with bone extracellular matrix and magnesium hydroxide, PLGA shows strong anti-inflammatory properties and osteoconductivity. This new product, PLGA-magnesium hydroxide-extracellular matrix, seems to accelerate human bone-marrow mesenchymal stem cells growth and development, local osteogenesis, and angiogenesis.[157]

Other research groups have focused on other compounds such as polyethylene glycol (PEG), which could be added to polylactic acid scaffolds to increase the osteoconductivity, facilitating molecular exchanges and bone marrow stromal cells traffic, ultimately promoting osteogenesis.[155] Rowland et al[156] have indicated that polydioxanone (PDO) may also be a good material for 3D scaffold manufacturing with increased osteogenic and adipogenic properties, but its entire range of effects are not known.

Some authors have focused their work on photo-crosslinked poly-propylene fumarate and diethyl fumarate scaffolds. This type of 3D bio-manufactured product has an increased stiffness, and remarkably allows a local overexpression of vascular endothelial growth factor (VEGF), bone morphogenic protein 2, transforming growth factor-β1 (TGF-β1), fibroblast growth factors-2 (FGF-2), and Runx2 transcriptional factor. Overall, all of these would promote differentiation of bone marrow stem cells into osteoblasts, which ultimately would commence the synthesising of bone.[162]

In addition, since the scaffolds must provide strong mechanical balance after transfer into native bone tissue, a variety of hard materials has been tested, including polyhydroxybutyrate-polyhydroxyvalerate, silk fibroin essence and nanohydroxyapatite.[151] Research conducted in a horse model of metacarpal bone defect, reported that a composite scaffold made of polyurethane, hydroxyapatite and decellularized bone, promotes wound healing with significant osteogenesis at 60 days after lesion.[158] In addition, recent reports indicate that polyurethanes may be used as smart memory scaffolds (SMS) which possess self-expanding and self-fitting properties, their shape depending on temperature and hydration.

However, these properties are not fully understood, and more data is required before the smart memory properties of polyurethane scaffolds are fully identified.[159]

Vascularisation methods

Although scaffold technology using 3D bioreactors has made significant progresses, there are still substantial issues relating to the in-built structures that facilitate exchanges between the synthetic scaffold and the surrounding bone, such as: dimension of pores, functional shape, and bordering area. The 3D additive manufacturing approach has been currently tested in fabrication of exoskeletons, bones, and cartilages scaffolds, but construction remains challenging.[138] Although, personalised approaches employing 3D bioprinting technology has made possible the inclusion of a wide range of growth factors stimulating bone regeneration. Considering the importance of angiogenesis-osteogenesis coupling, at the present time numerous groups are aiming to induce concomitant vasculogenesis, angiogenesis and osteogenesis in the setting of bone regeneration using a 3D bio-printed scaffold.[163] In this context, in vitro pre-vascularisation has been advocated by numerous research groups. While it is known that seeding and culturing numerous cellular types is challenging, vascularisation of 3D manufactured scaffolds represents the key element of creating a viable product for bone regeneration.[41] Goranov et al[164] attempted to manufacture a 3D scaffold characterised by separation of vascular and bone osteoprogenitor elements. This was achieved by applying a non-homogenous magnetic field, allowing independent manipulation of these two cellular populations. It is believed that separate handling of the two different cellular populations could facilitate an optimal development of viable vascularised bone tissue.[164] However, while this approach is possible in vitro, it should be replicated on a large scale in animal studies before final conclusions could be drawn.

For optimal vascularisation of a scaffold for bone regeneration, some authors suggest "transplant" of bone marrow stem cells simultaneously with bone morphogenic protein 2. Histological evaluation of such a 3D construct has reported robust vascularisation as well as osteogenesis, which has remained, remarkably, only in the bone compartment of the scaffold.[165] Interestingly, bone morphogenic protein 2, a member of the TGF-beta family, has a strong pro-osteogenic action, but at very high concentration increases bone resorption by activation of osteoclasts.[166]

The importance of vascularisation of the 3D bio printed construct was highlighted in a recent animal model study in which the authors evaluated a polycaprolactone scaffold embedded with bone morphogenic protein 2, which was used for vertical osteogenesis.[167] While the structure of the scaffold mimicked both native cortical bone as well as cancellous bone, the area replaced by new bone formation was very small despite upregulation of collagen 1A1, osteopontin, and osteocalcin, and an efficient release of bone morphogenic protein 2. However, the authors concluded that this was the result of poor vascularisation of the scaffold.[167]

As mimicking native osteogenesis appears to be vital in bone regeneration, Chai et al[168] produced in vitro a 3D porous titanium-based scaffold which seemingly sustains growth and development of human periosteum derived osteoprogenitor cells, laying down mineralised extracellular matrix.[168] While the results have been encouraging in the setting of cell-culture bioreactor experiments, the protocol has not been tested in other settings, and its utility is not known at the present time. Additionally, while this method concentrates on the functionality of bone forming elements, the osteoprogenitor cells, it largely ignores the aspect of vascularisation. Considering that the scaffold is made of titanium, it is highly unlikely that this approach will have wide application, but it may be used in selected cases associated with limited bone destruction. Similar studies evaluating vertical bone growth using 3D scaffold implants with micro-architecture characterised by porosity similar to trabecular bone, have indicated that after osteoblast seeding, a high level of osteocalcin, osteoprotegerin, morphogenic protein 2 and VEGF was recorded. However, osteogenesis was not improved even with the addition of extracellular matrix.[169] Therefore, we could speculate that this may be due to lack of blood supply.

To overcome all the fabrication issues, determined by the lack of vascularisation of 3D constructed scaffolds, a new tissue engineering approach combining the use of angiogenic growth factors and hydrogels has been proposed. Sharma D et al[22] showed that local delivery of VEGF has a positive effect on BRONJ healing. VEGF, administered in a combination with a Hydrogel (thiol-modified denatured collagen (Gelin-S), heparin thiol-modified hyaluronan, and a thiol-reactive cross linker, PEGDA (Extralink)), promoted not only angiogenesis, but also osteogenesis, since VEGF regulates in vitro osteoblastic action.[22] In 80% of cases the lesion resolved without any residual necrosis or bone sequestration. In addition, the hydrogels induced an anti-inflammatory action, promoting tissue regeneration and healing.[22] Histological and immunofluorescence assessments by Sharma D et al[22] found overexpression of CD105, a surface structure motif of the TGF beta receptor II, which is synthesised during new vessel formation, and von Willebrand factor, indicating efficient overall vascularisation.[22]

Gene expression studies confirmed the morphological and immune-florescence data.[22] Although these results suggest that a VEGF-hydrogel construct could be an adequate strategy for MRONJ therapy, the angiogenesis process, which is crucial for bone regeneration and healing, depends on the local vascular progenitors and cannot be controlled adequately. Again, poor angiogenesis represents a common and vital barrier to tissue regeneration.

In conclusion, vascularisation of 3D additive manufactured scaffolds constructs for bone regeneration is of paramount importance and while most of the studies have failed in providing reliable, robust, and efficient vascularisation and angiogenesis, it is now obvious that new bioengineering research should establish innovative methods of vascularisation including manipulation and transplantation of microvessels.

Chapter Three - HAMV: a new approach for bone regeneration

STATEMENT OF CONTRIBUTION TO CO-AUTHORED PUBLISHED PAPER

This chapter includes a co-authored paper. The bibliographic details of the co-authored paper, including all authors, are:

B Boston, D Ipe, A Gresita, S Hamlet, R Love, C Ling, I Nusem, RI Miroiu, PH Warnke, EB Petcu. Human adipose derived microvessels: a new approach for bone regeneration.

In preparation: final stages before submission

My contribution to the paper involved:

Providing direction on the scope and structure of the analysis, performing the search, assessing and screening the articles, preliminary analysis, and categorisation of the data into a usable format, drafting and editing the paper.

Signed [redacted] 14/10/21

Bridget Boston

Countersign [redacted] 15/10/21

Corresponding author of paper: Dr. Eugen Petcu

Countersigne [redacted] 16/10/21

Supervisor: Dr. Deepak Ipe

Introduction

Vascularisation is of paramount importance in numerous pathological conditions such as, cancer, diabetes, and neurodegenerative diseases, but also in healing and regeneration. In these settings, the amount of nutrient supply as well as the accumulation of metabolic residues are key factors that modulate the functionality of the local tissue. In regenerative medicine, one of the most important problems encountered in an experimental setting is represented by inability to modulate a functional vasculature that supports the biological or non-biological grafts. However, current bioengineering methods have focused on new modalities to control and handle local vascularisation.[19,28] In this context, new research has focused on understanding vascular architecture and its dynamics with the final aim of efficiently controlling its development. However, tissue engineering research, cannot utilise tissue samples that solely depended on diffusion for growth, as these samples cannot reach relevant dimensions required for optimal functionality.[40] Therefore externally modulating angiogenesis may lead to better results in experimental tissue engineering.

a. Adipose tissue and angiogenesis development

Manipulating vascular architecture including its form and function requires, among other elements, the exact measurement of vascular density in specific tissues, and in some specific areas, in the same tissue. However, an effective measurement of a dynamic vascular network, is difficult to realise, considering the three-dimensional architecture of blood vessels. However, a series of experiments, aimed to efficiently quantify the vascular pattern in angiogenesis models, using semi-automated machine learning methods, have returned promising results. BioSegment is a software, which can perform accurate vascular density measurements. The software performs vessel quantification, with high speed and accuracy, by assessing fluorescent images and even phase contrast images. This new technology could provide a highly efficient monitoring system, especially for experiments that require constant monitoring of developing blood vessels.[134]

Achieving an optimal vascular pattern and architecture is a dynamic process strongly influenced by environmental factors. Therefore, environmental, mechanical, and biophysical factors, or even the interaction with other cell populations, such as stromal cells, can deeply affect the development of new blood vessels.[32] Furthermore, the growth and development of these vessels requires several modifications and alterations to the surrounding cellular microenvironment. Reorganising surrounding stroma and alterations in the extracellular matrix is necessary for new vessels to develop into mature vascular structures.[32] For example, proteolytic enzymes act on the surrounding tissue and provide the necessary space needed for new vessels to expand, while a series of growth factors have the purpose of suppling the much-needed support for vessel development.[32] A series of stromal cells such as macrophages and mesenchymal stem cells intervene in this complex process to promote vessel sprouting. Additionally, further development requires vascular fusion and tissue remodelling.[32]

To establish new connections, newly formed capillaries require localisation of other vascular elements. This process often leads to neovessels crossing tissue boundaries and interfaces. Unfortunately, the exact method through which these vessels cross such structures, is not yet known.[135] However, studies that have used experimental angiogenesis models, in which the new vessels had to interact with a 3D interstitial collagen matrix and progenitor stem cells, reported that this interface acted as a barrier to vessel growth.[135] The new vessels grew parallel to the interface, proving that angiogenesis is paramount for establishment of a well-developed vascular network. It seems that the progenitor stem cell population provides positional guidelines to the growing neovessels and is therefore, highly important for efficient vessel and tissue development. Remarkably, local stromal stem cells also provide guidance points for the overall organisation and architecture of the microvasculature, within the tissue.[135] In adult humans, mesenchymal stem cells can still be isolated from the bone marrow and adipose tissue. A specialised cell population, made of CD11b+ and Gr1+ stromal stem cells, has been recently identified in adipose tissue and are considered as bona fide mesenchymal stem cells responsible for maintaining tissue homeostasis including angiogenesis.[111]

Considering the importance of adipose tissue as a source of elements for angiogenesis and vascularisation, numerous research groups have focused their work on this tissue. Initially, human adipose-derived stromal vascular fraction (hSVF) cells, were isolated and tested as they are easily accessible and more importantly, have the capability to form functional microvascular structures.[91] However the exact mechanism through which these cells are capable of self-assembling in mature vascular structures is not yet understood. Therefore, recent studies have utilised a series of angiogenesis inhibitors to assess the exact mechanism through which vascular re-assembly can be modulated. The results of these studies have proven that pan-Wnt inhibitor IWP2, produced significant inhibition of human adipose-derived stromal vascular fraction (hSVF) cells, but Wnt5a, produced by hSVF, plays an important regulatory role in microvascular self-assembly, ultimately suggesting that the self-reassembly of the microvascular structures is modulated by this molecular pathway.[91]

Therefore, under modulation from Wnt pathway, human adipose-derived stromal vascular fraction (hSVF) cells can promote the production of neovascular structures at the location where they are transplanted. On the other hand, several research experiments have reported that angiogenesis and overall vascularisation depend not only on the quantity of the cells being used but also the age of the donor.[9]

A recent study, carried out on two cohorts of laboratory animals investigated this theory using stromal vascular fraction (SVF) enzymatically isolated from the adipose tissue of young (ySVF, 4 months) and old (oSVF, 24 months) Fisher-344 rats. The SVF was combined with type I collagen and then implanted subcutaneously into young Rag1 mice. The results were evaluated at either two or four weeks after the implant was performed.[9] The study concluded that regardless of the age of the donor, both ySVF and oSVF have the same viability, proliferation, migration rate and even the same tube formation, at 2 weeks post implantation.[9] However, at 4 weeks, the differences between the two groups were notable. More specifically, the structural characteristics of the neovesels in the ySVF group were better than in the oSVF group. This result was backed up by blocking thrombospondin-1 (Thbs-1) which is greatly expressed in old, but not in young, groups. This led to a decrease in the structural and functional differences between the two groups.[9] Therefore, it seems that the age of the donor does not affect initial angiogenesis but does eventually impair the creation of a mature microcirculation. This outcome can however be significantly reduced by modulating Thbs-1.[9]

Human adipose-derived stromal vascular fraction (hSVF) has also been used successfully as a method of microvascular protection in a model of murine myocardial infarction.[29] Coronary blood flow and left ventricle function were evaluated and closely monitored after myocardial infarction in groups of mice which either received a SVF injection or did not. The study reported that implanting SVF constructs, immediately after myocardial infarction, promotes and maintains heart function.[29] Furthermore, microvascular perfusion in the affected area is sustained by promoting coronary blood flow.[29] Other studies have even tested the SVF therapy for an established myocardial infarction. SVF cells, isolated from rat adipose tissue were implanted in the affected area. The studies concluded that SVF implants can stabilise the myocardial tissue at time of administration. Cardiac performance is maintained at that level, without any further decrease in function, while the infarct volume also remains roughly unmodified. Increased microvessels perfusion of the infarct area, seems to be the mechanism responsible for this result.[30] Nonetheless, at the present time, the optimal amount of SVF required to sustain the development of a robust vascular network is not known but it seems that a key element is represented by SVF embedding into collagen gels.[39]

Additionally, in vitro mechanical factors can modulate angiogenesis and maintain the viability of newly formed blood vessels.[161] In this context, a series of studies have tested different stress fields during vascular growth to induce specific three-dimensional microvessels constructs. These experiments indicated that vessel architecture can be modulated in vitro, and microvessels usually align perpendicular to the direction of compressive strain. Obtaining control of vasculature development in experimental engineering technics represents a breakthrough for future experimental and therapeutic options.[161]

Mechanical interactions between the extracellular matrix and neovessels are responsible for angiogenesis development. Although, this process is not completely understood recent studies have reported that newly formed blood vessels directly interact with the interstitial matrix which serves as a guide, during their growth and development. Therefore, it can be determined that angiogenesis and development of the local vascular network is modulated by compressive strain during dynamic tissue deformation.[38] The viscoelastic structure of the local collagen allows vessel deformations to take place. Remarkably, these compressive strain forces, further continue after the inception of angiogenesis, and contribute to the development of the final mature vascular network. However, pre-existing vascular architecture has no significant effect on the development of new vascular structures.[38]

Angiogenesis development depends on interactions between newly formed vascular sprouts and the extracellular matrix. However, the mechanisms controlling the relationship between mechanical and biophysical properties of the ECM and neovessel growth during sprouting angiogenesis are generally not understood. A study which assessed the interactions between vessels and extracellular matrix indicated that modifications made to individual neovessels can produce an overall change in the final vessel architecture.[26] Interestingly, a higher collagen density in the extracellular matrix induces development of shorter vessels with less branch points, consequently vessel interconnectivity is significantly diminished.[26] The study also concluded that an increase in density of the extracellular matrix is responsible for a severe decrease in angiogenetic processes within the 3D organ culture model. The stiffness of the extracellular matrix is apparently responsible for this outcome, which eventually diminishes the neovessel growth rate and expansion, leading to modest results. [26]

b. HAMV and tissue regeneration

At the present time there is no data from similar experiments conducted using human adipose-derived stromal vascular fraction (hSVF) in the setting of bone necrosis or destruction in which tissue regeneration is required. The angiogenic and vasculogenic capabilities of human adipose-derived stromal vascular fraction (hSVF), should be tested in other ischemic pathologies and regenerative medicine experiments particularly involving bone healing and regeneration. However, the mere existence of new blood vessels is not sufficient for tissue growth and development.[161]

Hoying et al[31] have pioneered an improved approach in these type of experiments by developing and testing a more efficient version of human adipose-derived stromal vascular fraction (hSVF), the human adipose derived microvessels. Their initial research indicates that that similar to microvessels isolated from adipose tissue of rats, HAMV may be cultured. In fact, the HAMV represent fragments of human vasculature, retaining key elements, such as endothelial progenitors and endothelial cells, pericytes, stem cells and macrophages, which have a significant role in the formation of a new vascular network to subsequently promote tissue regeneration.[31] These human microvascular fragments displayed characteristics of arterioles and venules, with pericytes and smooth muscle cells, and were embedded in an extracellular matrix composed of collagen type I, III and IV. The viability studies showed that the 3D microvessels were capable of proliferation, migration, and elongation, proportional in magnitude to the initial vascular density. Interestingly, an accelerated elongation was observed after addition of VEGF.[31]

Rauff et al[32] indicated that newly formed blood vessels, derived from the 3D vascular constructs of Hoying et al[31], are capable of releasing proteolytic enzymes which degrade their own basement membrane, thus facilitating vascular sprouting and growth.[32] The development of the vascular network is further promoted by the ability of the endothelial cells to migrate, which is determined by an inter-play between pull and push traction forces and contraction, resulting in a deformation of matrix.[33,34,32] These neovessels also react to various extracellular matrix factors such as, VEGF, fibroblast growth factor (FGF), tumour necrosis alpha (TNF-alpha) and angiopoietins (Ang). In parallel, the mesenchymal stem cells, as well as macrophages, facilitate vascular sprouting, fusion, and remodelling, via secretion of matrix metalloproteinases, which, through their local action, enable the development of vascular networks (MMP). Overall, these events are of paramount importance for healing and regeneration, including organ morphogenesis and biomaterial tissue development.[35,36,37,38] Additionally, functional capillaries, such as those provided by the adipose tissue, are superior to any type of transplanted progenitor stem cell. Recent studies evaluating the regeneration of infarcted hearts has shown that transplanted microvessels provide a better chance of recovery than pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs), as a significant number of these often die shortly after transplantation.[170] Moreover, HAMV have been successfully used in an experimental model of type 1 diabetes therapy to sustain the transplant of pancreatic islet cells. After HAMV co-transplantation, glucose tolerance and overall diabetes symptomatology improved significantly in 3 types of experimental type 1 diabetes.[160]

Based on this HAMV method, vascular tissue organoids have been developed. Organoid models replicate the interactions found in native tissue with high fidelity and do not require the sacrifice of animal models, making them extremely valuable for modern research.

HAMV incorporated into organoids has recently shown significant pro-angiogenic potential. In this construct, the microvessels maintain both their physical and cellular structure, proving to be a better model of vascular network development.[134] Remarkably, angiogenesis is promoted thorough the organoid structure, expanding externally when a 3D collagen matrix is introduced. However, the growth of new vessels is strongly dependent on the vascular connectivity between adjacent vessel beds.[134]

In conclusion, the HAMV represent a superior approach for tissue healing and regeneration which theoretically could be used in numerous pathological settings which involve necrosis or immense tissue destruction. Although it has been established that an adipose-derived stromal vascular fraction (hSVF) has a positive role in tissue regeneration, HAMV is superior, as the induced angiogenesis is robust, offering theoretically better chances for development of a functional vascular network, ultimately producing a better outcome, especially if incorporated in vascular tissue organoids.

Chapter Four – Material and Methods

HAMV cell culture

Microvessels prepared from human adipose tissue were supplied by Advanced Solutions Life Sciences (500 N Commercial Street, Manchester, NH 03101, USA). The HAMV were stored in liquid nitrogen for optimal long-term storage. When needed, frozen vials of HAMV were thawed and suspended in collagen gels for culture using the following protocols.

Reagent Preparation

4X DMEM

- 10g of powdered DMEM (low glucose w/ phenol red; Fisher Scientific #31600-034)
- 3.7g of sodium bicarbonate (Fisher Scientific #S233-3)
- 10ml 1M HEPES (Lonza #17-737E)

Components were mixed until dissolved and brought to a final volume of 250ml in milliQ water. The mix was put through a sterile 0.22 µm filter.

Microvessel Culture Medium

A stock solution of VEGF was prepared. More specifically, 0.1% BSA was added to UltraPure water and filtered through a sterile filter. VEGF was then reconstituted with the sterile solution to achieve a final concentration of 10 µg/ml.

Collagen I

- 1 M NaOH
- Rat tail collagen I, high concentration (Corning® #354249).
- UltraPure or MilliQ water (sterilised by autoclaving or sterile filtering)
- 4x DMEM (see above preparation instructions)

A Collagen solution was made for each experiment. Detailed instructions can be found at step 4 of the procedure.

Thawing medium

- RPMI containing 10% fetal bovine serum (FBS)

Procedure

1. The total number of microvessels and volume of collagen/microvessel suspension required was determined using the following factors.

- Microvessels are routinely used at a density of 50,000 - 150,000 microvessels/ml for robust angiogenesis.
- Effective collagen concentrations range from 1.5 mg/ml to 7 mg/ml, with 3 mg/ml being the most frequently used concentration.
- Typically, 100µl of collagen/MV suspension is used for wells of a 96 well plate, and 250µl for a 48 well plate.

2. Microvessels were quickly thawed in a 37°C water bath and moved to a centrifuge tube containing approximately 10 ml of thawing medium. A micropipette and culture medium were used to rinse out the vial.

3. Microvessel suspension was centrifuged at 400g for 4 minutes.

4. While microvessels were in the centrifuge, the collagen gel was prepared with the following considerations.

- All components of the collagen mix were kept on ice throughout the preparation. Chilled pipette tips were also used to help prevent collagen from gelling.
- Extra collagen was prepared (approximately 50% extra) to compensate for tube and pipette wall adhesion of the viscous collagen.
- Once prepared, the collagen was used within 30 minutes.
- Collagen was prepared from 4x DMEM, sterile UltraPure water, and high concentration stock collagen. All the reagents were added together in a 15ml centrifuge tube. The collagen stock was the last reagent added.
- As the color was orange/yellow, a few microliters of sterile 1N NaOH was added until the mixture turned red/light pink (reflecting pH 7.4).

5. The supernatant was aspirated from the microvessel pellet so that no more than 50µl of media remained above the pellet. The microvessels were resuspended with a micropipette and the centrifuge tube was placed on ice for minimum of one minute, in order to cool the tube to prevent collagen from gelling in it.

6. The appropriate volume of cold collagen solution was dispensed into the tube containing the microvessel pellet and the pellet was resuspended thoroughly using a micropipette.

7. The microvessel/collagen mix was dispensed onto the appropriate plate.

8. The plate was placed in a 37°C, 5% CO_2 incubator for 1 hour to gel the collagen.

9. Culture medium was added to each well, at 1.5 times the volume of collagen in the well. The well-plate was placed back into incubator. Medium was changed every 4 days; as more frequent medium changes could have slowed neovessel growth.

For all experiments and culture of HAMV, frozen vials were thawed and seeded into 24-well tissue culture plates at a density of 1 x 10^4 cells/well with cRPMI supplemented with 50ng/ml VEGF, 1X B27 and 3mg/ml of collagen I.[41] Cells were cultured in a humidified incubator at 37°C, using a standard mixture of 95% air and 5% CO_2. HAMV were observed every 2-3 days under a light microscope and fresh media was provided as required.

HAMV viability and proliferation studies

AlamarBlue® assays were performed to evaluate the proliferation of HAMV according to the manufacturer's instructions (Invitrogen, Paisley, United Kingdom). HAMV were seeded in a 48 well plate at a density of approximately 10,000 cells/well. At each desired time point (Day 1, 5, 7, 14, 21 and 28) the fluorescence intensity of HAMV with 1/10 alamarBlue/culture media that were incubated for 1hr was measured using a microplate reader (POLARstar Omega plate reader, BMG Labtech) at EX544/590nm.

HAMV microscopy studies

To visualise any morphological changes in the HAMV, including cell death, a LIVE/DEAD® assay was performed on day 5 and day 28, and results visualised using confocal microscopy. For this, the cells were incubated at room temperature for 30 min with 1 µg/mL of Fluorescein Diacetate (FDA, Thermo Fisher Scientific, Australia) to visualise live cells (green) and 2 µg/mL of Propidium Iodide (PI, Thermo Fisher Scientific, Australia) to observe dead cells (red). The samples were then imaged at room temperature using a Nikon eclipse Ti confocal Microscope at Ex/Em 488/526 nm for FDA and Ex/Em 493/636 nm for PI at 10X magnification.

Osteoblast cell culture

Frozen stock of MG-63, a human osteoblast cell-line, were provided by Dr. Ipe, Griffith Health centre. In this study, MG-63 cells were cultured as previously described.[133] Briefly, a complete RPMI (cRPMI) media was prepared by adding 10% fetal bovine serum, 1% penicillin streptomycin, 1mM Sodium pyruvate, 1mM MEM Non-Essential Amino Acids Solution and

1mM HEPES to RPMI 1640 media. All reagents were purchased from Thermoscientific, Australia. Cells were cultured without any stimulatory supplements or vitamins in a T75 flasks (NUNC, Thermoscientific) in a humidified incubator at 37°C, using a standard mixture of 95% air and 5% CO_2. The culture media was replaced every 3-4 days. After cells reached approximately 90% confluence, cells were detached with 1X Trypsin and subsequently replated with fresh cRPMI medium (Figure S1.1; S1.2).

Osteoblast/HAMV indirect co-culture

For this study, the human osteoblast cell line MG63 was used at a density of 2.5 x 10^4 cells/well. Cells were seeded in triplicate into a 48 well plate with 500ul of conditioned media which was derived from the HAMV cultures (day 1 to 28). Standard fresh HAMV media (as described above) was used for MG63 control wells for each desired time points. qPCR, cell viability and morphological changes were subsequently assessed at desired time points. Cell viability and morphological changes were recorded using a common cell-culture inverted microscope (S1.3; S1.4; S1.5).

Quantitative real-time reverse transcription PCR (qRT-PCR)

Conditioned media derived from the HAMV cultures (Microvessel Cultured Media - MCM) was added (500 µl/well) to the osteoblasts (2.5 x 10^4 cells/well) to examine the influence of MCM secreted soluble factors on osteoblast differentiation. Osteoblast cells with normal microvessel culture media (NCM) were used as control wells/samples. Cells were exposed to MCM or NCM for up to 14 days.

For quantitative real-time reverse transcription PCR (qRT-PCR) experiments, RNA was extracted from these osteoblast cells cultured either in MCM or NCM using RNA extraction kits (RNeasy Mini Kit, QIAGEN, USA) on Day 7 and Day 14. RNA quality was checked by spectroscopy (nanodrop), and total RNA (1µg) was reverse transcribed to cDNA using the SensiFAST™ cDNA Synthesis kit from Bioline (Bioline, Aust Pty. Ltd Australia) following the manufacturer's instructions. Forward and reverse primers used to determine the expression of desired genes are described in Table 1.

Table 1. Forward and reverse primers used to determine the expression of desired genes

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
ALPL	CCAAGGACGCTGGGAAATCT	TATGCATGAGCTGGTAGGCG
BMP2	TGTATCGCAGGCACTCAGGTCA	CCACTCGTTTCTGGTAGTTCTTC
OCN	CTCACACTCCTCGCCCTA	GGTCTCTTCACTACCTCGCTG
VEGF	GAGCCTTGCCTTGCTGCTCTA	CACCAGGGTCTCGATTGGATG
WNT5a	TACGAGAGTGCTCGCATCCTCA	TGTCTTCAGGCTACATGAGCCG
ACTB	CACCATTGGCAATGAGCGGTTC	AGGTCTTTGCGGATGTCCACGT

Real-time quantification of transcripts was performed using Bioline SensiFAST SYBR No-ROX kits (Bioline, Aust Pty. Ltd Australia), following the manufacturer's instructions in an Applied Biosystems™ QuantStudio™ 6 Flex Real-Time PCR System with 96 well plates. PCR conditions were: initial denaturation at 95°C for 2min, and 40 cycles of: 95° C for 5 sec, 60° C for 10 sec and 72° C for 5 sec. All reactions were performed in triplicate and expression values were normalised using reference gene - actin (ACTB). Relative fold change values were calculated using amplification efficiencies, as described previously.[88]

Statistical Analysis

Quantitative data will be expressed as means ± standard deviation. Comparison between groups will be analysed by one-way analysis of variance with Sidak's multiple comparisons post hoc testing using GraphPad Prism 8.0 software. Mean differences with a p value of < 0.05 will be considered to be statistically significant.

Chapter Five – Results

Proliferation assay

In order to evaluate the proliferation of the HAMV, AlamarBlue assays were performed. Initial proliferation assay showed robust proliferation and outgrowth from two weeks, as seen in Figure 8.

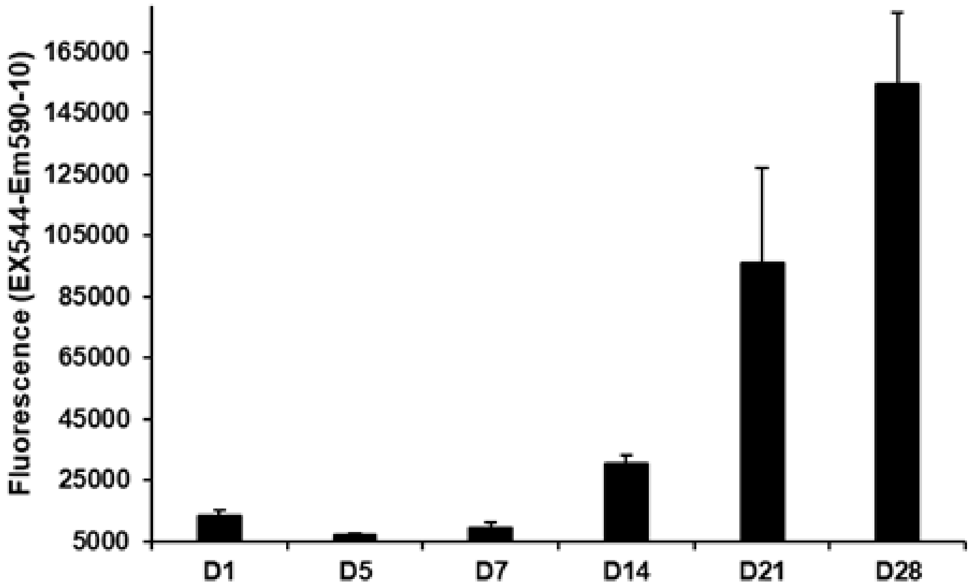


Figure 8. HAMV viability and proliferation demonstrating growth of HAMV (10,000 cells) in a 3D collagen matrix over one month.

Fluorescence intensity of HAMV after 1h incubation with alamarBlue at days 1, 5, 7, 14, 21 and 28 post seeding. The number of HAMV decreased during first week in culture, then exponentially increased. Data points are expressed as mean values ± SD.

HAMV in vitro growth

To qualitatively analyse and visualise any morphological changes in the HAMV, including cell death, a LIVE/DEAD® assay was performed on day 5 and day 28. Results were visualised by confocal microscopy and showed robust growth by day 28 as shown in Figure 9, which was consistent with the previous alamarBlue quantitative results.

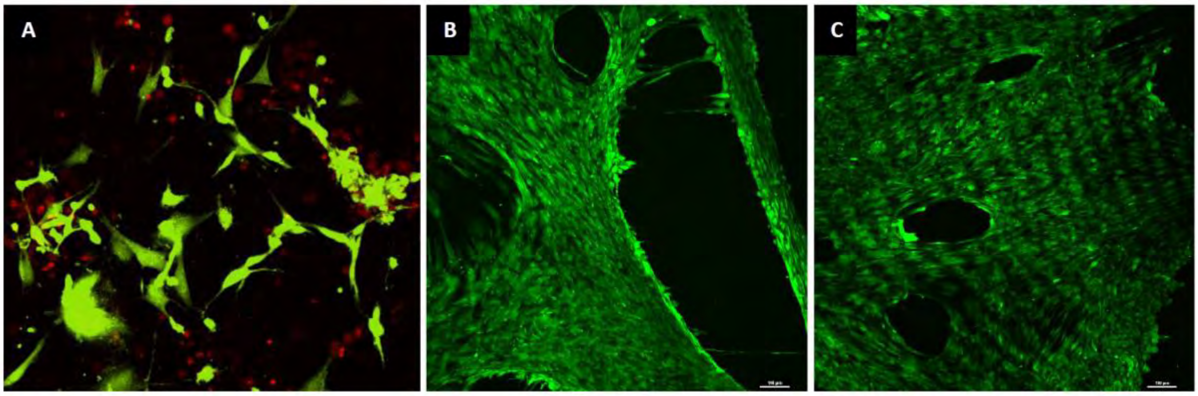


Figure 9. HAMV in vitro growth: dynamics of tubular structure development.

A. Day 5. B. Day 28. C. Sheet-like 3D tubular structure formation at day 28. A LIVE/DEAD® assay was performed on day 5 and day 28, and results visualised under Nikon eclipse Ti confocal Microscope at Ex/Em 488/526 nm for FDA and Ex/Em 493/636 nm for PI at 10X magnification. Live cells exhibited green florescence whereas dead cells, red. The percentage of green fluorescent (live cells) progressively increased over the month, forming a well-defined mesh-like structural network. Scale bars represent 100 micrometres.

Osteoblast differentiation - qPCR results

Changes in the relative expression of osteoblast differentiation markers during culture with HAMV conditioned media was examined at days 7 and 14. Overall, the expression of osteoblast differentiation marker genes was greater at day 7 compared to day 14. All results are compared to expression levels in normal media. qPCR data levels were normalised relative to the expression of the housekeeping gene beta-actin, which was used as an internal control. Error bars indicate standard errors of the means. One-way analysis of variance (ANOVA) with Sidak's multiple comparisons post-hoc testing was applied to determine the significance of any differences in mean values. *$P < 0.05$, **$P < 0.01$ versus day 7. Results are shown in Figure 10.1, 10.2, 10.3, 10.4 and 10.5.

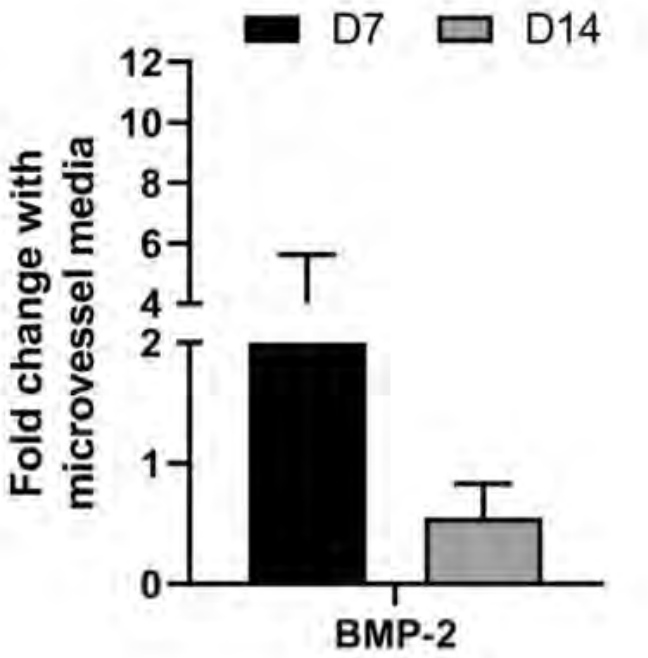


Figure 10.1. Expression of the osteoblast marker bone morphogenetic protein-2 (BMP-2) at day 7 and day 14.

BMP-2, a bone morphogenetic protein, plays an important role in the development of bone and cartilage. Relative expression levels of BMP-2 were measured at day 7 and day 14. qPCR results showed BMP-2 expression levels decreased from day 7 to day 14.

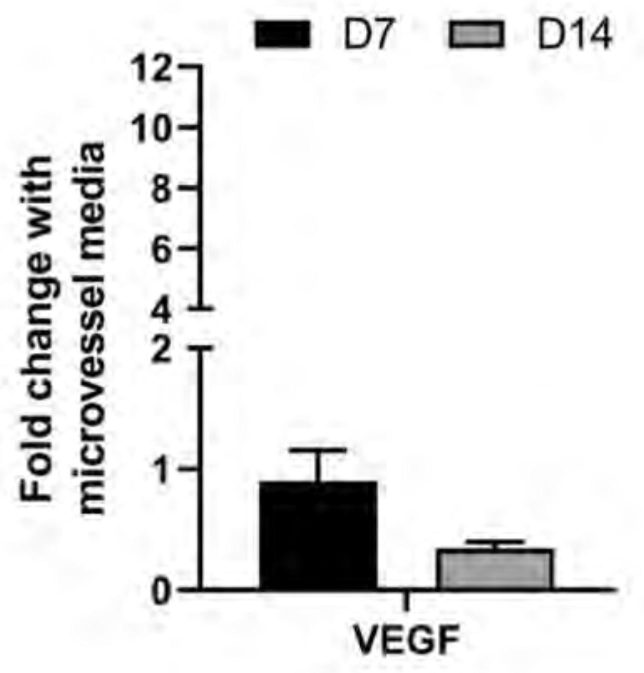


Figure 10.2. Expression of the osteoblast marker VEGF at day 7 and day 14.

VEGF expression did not vary significantly over the entire period, displaying the lowest fold change decrease between day 7 and day 14.

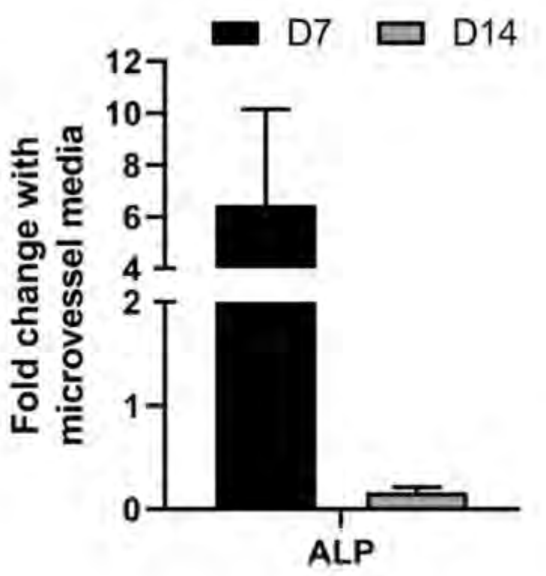


Figure 10.3. Expression of osteoblast marker alkaline phosphatase (ALP) at day 7 and day 14.

ALP, alkaline phosphatase, an enzyme responsible for the growth and development of bones, demonstrated significantly high expression on day 7, compared to day 14 (Sidak's multiple comparisons test: $p<0.0094$).

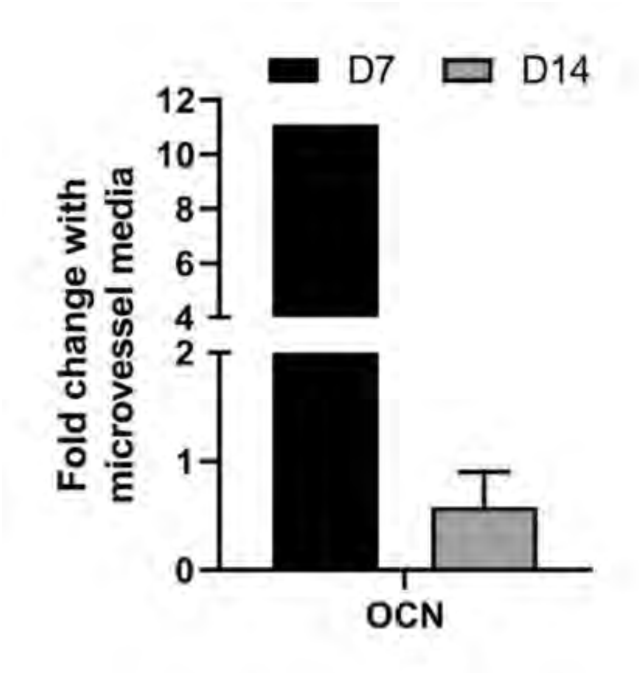


Figure 10.4. Expression of osteoblast marker Osteocalcin (OCN) at day 7 and day 14.

Expression of mineralisation-specific gene, Osteocalcin, was again significantly greater at day 7 compared with day 14 (Sidak's multiple comparisons test: $p < 0.0007$).

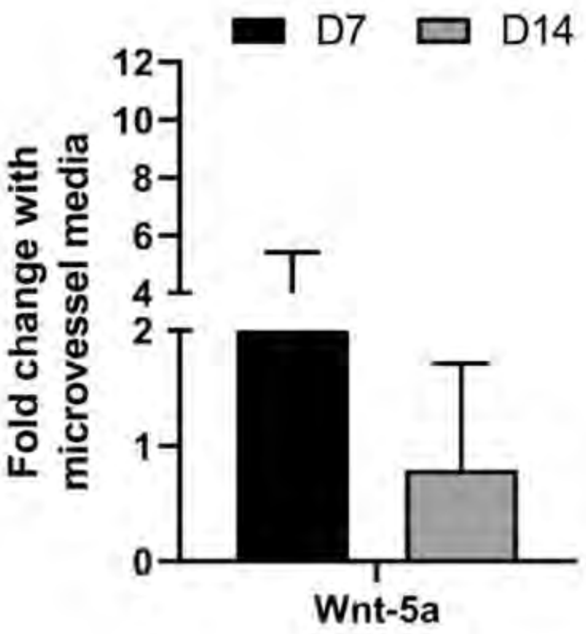


Figure 10 5. Expression of osteoblast marker Wnt-5a at day 7 and day 14.

Time course qPCR expression analysis of bone-related gene, Wnt-5a at day 7, and day 14. For day 7, significantly greater expression was found compared to day 14.

Table 2. Sidak's multiple comparisons test

D7 - D14	Predicted (LS) mean diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
ALP	6.29	1.35 to 11.2	Yes	**	0.0094
BMP-2	1.75	-2.68 to 6.17	No	ns	0.7932
OCN	10.5	4.26 to 16.8	Yes	***	0.0007
VEGF	0.557	-3.87 to 4.98	No	ns	0.9983
Wnt-5a	1.31	-3.11 to 5.74	No	ns	0.9244

Alpha = 0.05

Chapter Six - Analysis and Discussion of Results

Evaluation of HAMV proliferation in 3D collagen matrix

Our study has shown that human adipose derived microvessels (HAMV) can proliferate efficiently during in vitro cell culture. The HAMV preparation represents fragments of human vasculature retaining key elements such as endothelial progenitor and endothelial cells, pericytes, stem cells, myeloid progenitors and macrophages, which all have a significant role in the inception of a new vascular network during tissue regeneration.

This confirms the previous research conducted by Hoying et al[31], which cultured microvessels isolated from the adipose tissue of rats. Hoying el al[31] had many important findings which formed the foundation of our HAMV methodology, in particular, that microvasculature expands subsequent to cellular and extracellular influences. The fresh rat adipose derived microvessels develop in vitro in the presence of collagen I and close to the extracellular matrix. The authors revealed not only collagen I, but also collagens III and IV, are important for the development and growth of microvessels.

Therefore, we used collagen I in our study, as well as the serum-free culture supplement 'B-27.' B-27 was initially developed for neuronal cell culture, yet now it has been widely adapted and successfully used in many other applications, including for the growth and maintenance of various cells in 3D cell culture. However, most importantly, our cell culture media included VEGF, the most powerful pro-angiogenic factor, inducing and sustaining growth and expansion of HAMV. In fact, with some limitation, we believe that our protocol reiterates the vascular growth and development which usually occurs in vivo. After an initial decrease in the number of viable vascular structures, which represent the elements that have not survived the storage at -80^{0}C and the subsequent handling / establishment of cell culture, the number of microvessels increases logarithmically from day 7 to day 28, suggesting a sustained development (Figure 8).

Assessment of HAMV growth and morphological changes

A LIVE/DEAD® assay was performed on day 5 and day 28 to assess any structural or morphological changes. Results were consistent with the alamarBlue assay results, showing both live green florescent vessels and some dead red florescent vessels at day 5, and significant robust growth by day 28 (Figure 9). The percentage of green fluorescent live microvessels increased progressively over the month, forming a well-defined mesh-like structural network by day 28. This sheet-like 3D tubular structure formation is consistent with HAMV morphology and is a feature indispensable for proper vascular perfusion. Remodelling of the surrounding matrix in this way is essential for promoting structural integrity, initiating angiogenesis and subsequent vascular expansion. Overall, the HAMV-Hydrogel 3D collagen construct was found to be suitable for long-term cell culture, based on tests for cell viability and proliferation.

Assessment of osteogenic gene expression by qPCR

During foetal development, an established vascular network precedes osteogenesis. This raises the possibility that microvascular endothelial cells secrete factors that play a role in the regulation of osteogenesis. To determine whether any soluble factors affecting osteoblast differentiation are secreted by HAMV, the osteoblasts were exposed to up to 14 days treatment with HAMV conditioned culture medium. We demonstrated a paracrine effect of the HAMV on the differentiation of osteoblasts using quantitative-PCR. Growth factors such as Bone morphogenetic protein 2 (BMP-2) and vascular endothelial growth factor (VEGF) are essential during the process of angiogenesis and play critical roles in bone formation. Other osteoblast differentiation markers such as alkaline phosphatase (ALP) and osteocalcin (OCN), as well as the osteogenic regulator Wnt-5a, were also used to assess the effect of HAMV secreted factors on osteoblast differentiation.

We investigated the role of BMP-2 in osteogenic differentiation at day 7 and day 14. Bone morphogenetic proteins (BMPs) regulate proliferation and/or differentiation of osteoblasts and chondrocytes. BMP2 was originally identified as an inducer of bone and cartilage formation.[136] Prior studies have proved that BMP-2 is involved in bone formation, bone remodelling, bone development, and osteoblast differentiation.[136] Our study showed significant differences in the expression of m-RNAs for the gene BMP-2 in osteoblasts between the conditioned media and normal media. The expression of BMP-2 in conditioned media was initially significantly higher compared to the normal media control (Figure 10.1). More specifically, a 2-fold change for conditioned media was observed on day 7. By day 14 however, BMP-2 expression decreased in the HAMV-treated osteoblasts (0.5-fold change). Results of descriptive statistics also show that expression of m-RNA for BMP-2 is higher at day 7 than day 14.

Recent evidence indicates that VEGF is an important regulator of osteogenesis, mediating bone vascularisation and differentiation towards osteoblasts.[137] Here, we detected VEGF gene expression in cells committed to osteogenesis, by conditioned media and normal media. Although HAMV-conditioned media was able to induce VEGF gene expression at a higher level than normal media, the effect was not significantly greater in magnitude (Figure 10.2). In fact, not only did VEGF expression in conditioned media not vary significantly from normal media, but VEGF expression also didn't differ considerably over the time period and displayed the lowest fold change decrease between day 7 and day 14.

Another gene which showed higher differential expression in HAMV-conditioned media compared to normal media, was alkaline phosphatase (ALP), a protein associated with osteoblast cell phenotype. As known in the literature, post proliferation differentiating cells become alkaline phosphatase (ALP) positive and activity increases as the culture progresses towards the mineralisation phase. As detected by qPCR analysis, the expression of ALP was 6 times higher in cells differentiating in conditioned media compared to normal media, which suggests a higher differentiation rate because of the influence of microvessels (Figure 10.3). Additionally, expression m-RNA for ALP was lower at day 14 compared with day 7. This finding was consistent with another study[171] in which initially high ALP expression continued to decrease over 28 days. As the protein ALP is not only an osteoblast differentiation marker but also an undifferentiation marker, the study suggests expression was high on day 0 because cells were undifferentiated, then declined over time before it exhibited an increasing trend again after day 28, due to osteoblast differentiation.[171]

OCN is another typical osteoblast function marker, playing an important role in osteogenesis. More specifically, OCN is a 'late-stage' osteoblastic differentiation marker. In the current study, we observed an elevated expression of OCN at day 7 versus day 14, compared to normal media (Figure 10.4). The significant increase of 11-fold compared to normal media values was observed (p value < 0.0007).

Wnt regulates bone formation through β-catenin-dependent canonical and -independent noncanonical signalling pathways. More specifically, Wnt-5a is expressed early on during in vitro osteogenesis, promoting the differentiation of mesenchymal stem cells into osteoblast lineage cells. Similar to the previous differentiation markers, we observed elevated expression of Wnt-5a at day 7 versus day 14, compared to normal media (Figure 10.5).

A consistent observation from the qPCR analyses is the initial increase in the expression of osteogenic differentiation markers by day 7 followed by a decrease over the following 7 days. This may be due to a number of factors outside the scope of this study, which, unfortunately, will need further investigation to completely delineate. A decrease in the availability of differentiation stimulatory factors such as VEGF in the HAMV-conditioned media by day 14 is unlikely, given the alamarBlue proliferation results, which showed increasing microvessel numbers at that time.

However, the consistency in this result may suggest a common temporal response as measured by gene expression during osteoblast differentiation. For example, a high throughput microarray study of human MSC's following BMP6 stimulation which covered early to late stage osteoblast differentiation and mineralisation, showed that while varying sets of biological processes and functional gene groups were involved at different stages of osteoblast differentiation and mineralisation, gene expression was maximal by day 4 decreasing significantly by day 10.[172] Notwithstanding the obvious differences in this study, it does however provide some evidence to suggest that the decrease in the expression of osteogenic markers at day 14 was perhaps not unwarranted.

Furthermore, the osteoblasts used in this study were the well-defined human osteosarcoma cell line MG-, and not primary osteoblasts. Although osteosarcoma-derived cells are commonly used as osteoblastic models, their molecular malfunctions are still not completely understood. For example, studies have shown that different osteosarcoma-derived cell lines such as MG-63, Saos-2, and U-2 OS cells are more heterogeneous compared to human osteoblasts for almost every protein investigated.[173] This demonstrates the importance of examination techniques at the single cell level, and further emphasises the limits of techniques that require lysis of cell aliquots and homogenisation of their mRNA transcripts as used in this study. Although RT-PCR phenotyping has been extensively performed for MG-63, Saos-2 and U-2 OS cell lines, these studies have not addressed what proportions of cells produce the transcripts, and whether subpopulations exist.[174,175]

Overall, the results demonstrating the ability of the HAMV-conditioned medium to optimise the osteogenic potential of osteoblasts was confirmed by qPCR analysis of osteogenic differentiation marker genes, showing that the gene expression program modulated by this medium was translated into a phenotypic osteogenic commitment at the intact cell level.

Chapter Seven - Conclusions

This study has shown that human adipose derived microvessels (HAMV) are viable after long-periods of storage in liquid nitrogen. HAMV expanded exponentially in a collagen I hydrogel cell culture system, reaching a maximum of growth and development after four weeks. Morphological characterisation by confocal microscopy indicated that HAMV form a well-developed expansile mesh-like vascular network. This is an important finding demonstrating not only the viability of these structures, but also their tremendous ability to grow and develop in vitro, ultimately enabling their use for tissue regeneration applications. These findings must also be tested in an animal model, but it is now clear that the mesh-like structure could provide a better chance for tissue re-vascularisation and subsequent healing and regeneration.

Although the exact architectural topology has not been investigated on this occasion, HAMV can replicate all the steps described during vasculogenesis and angiogenesis, including sprouting and branching. McCoy et al[176] suggest that collagen I might be the most important factor modulating the significant development of a vascular work, being the key element identified in the composition of the extracellular matrix and capillaries. It is known that inhibition of collagen would impair angiogenesis.[177,178]

However, our morphological evaluation of HAMV indicates that these products are efficient and should be tested in association with other bioengineering materials, such as hydrogels, as the number and density of the newly formed vascular tubular structures can provide the optimum environment for seeding various type of progenitor and stem cells in combination with various growth factors.

Remarkably, this study has also revealed a significant paracrine effect of the HAMV on osteoblasts. More specifically, after two-weeks, the gene expression of BMP2, the most important modulator of osteogenesis and chondrogenesis, showed a substantial decrease in osteoblast treated HAMV. The same trend was recorded for ALP. Also, lower levels of OCN, a marker of osteoblastic differentiation, as well as Wnt, a significant molecular pathway of modulating osteogenesis, were increased after one week, but considerably decreased at day 14. Conversely, HAMV conditioned media promoted overexpression of VEGF, but this was not considered to be significantly different to that of the control, in which normal media was used. Interestingly, the VEGF level quantified by molecular methods did not match morphological changes, which indicated a strong development of a mesh-like vascular network. These changes may be explained by heterogeneity of the MG-63 osteoblast cell lines, suggesting that for future research perhaps single cell evaluations should be used.

Overall, these results indicate that HAMV-conditioned medium has the ability to modulate the osteogenic potential of osteoblasts. Ultimately this suggests that HAMV can be used as a viable tool in various regenerative medicine experiments, especially if used simultaneously with extracellular matrix components such as collagen I, and pro-angiogenic factors such as VEGF. Integration of all these elements in a new construct could offer a new efficient therapy of MRONJ and ultimately a new improved approach in tissue regeneration.

Appendices

Appendix 1

S1 Supplementary information for chapter four.

S1.1 Osteoblasts were trypsinsed and counted at day 21 using a hemocytometer.

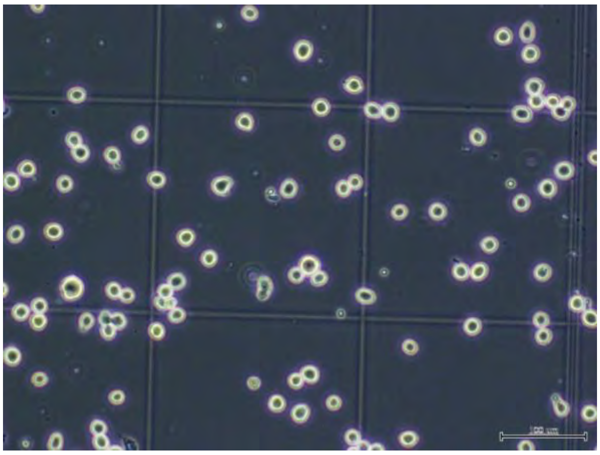


Figure S1.1 Osteoblasts - day 21 under hemocytometer.

S1.2 Osteoblast cells seeded in T75 flask for culturing purpose (90% confluent).

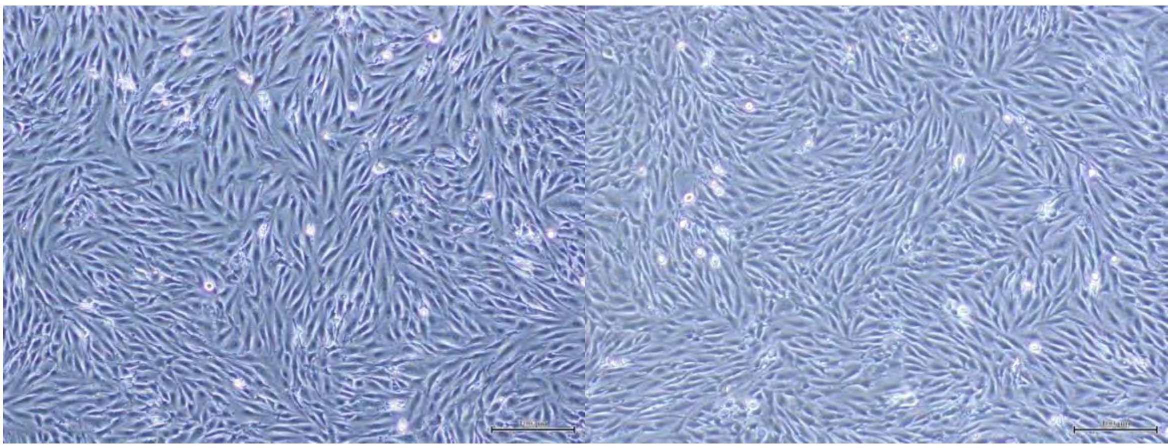
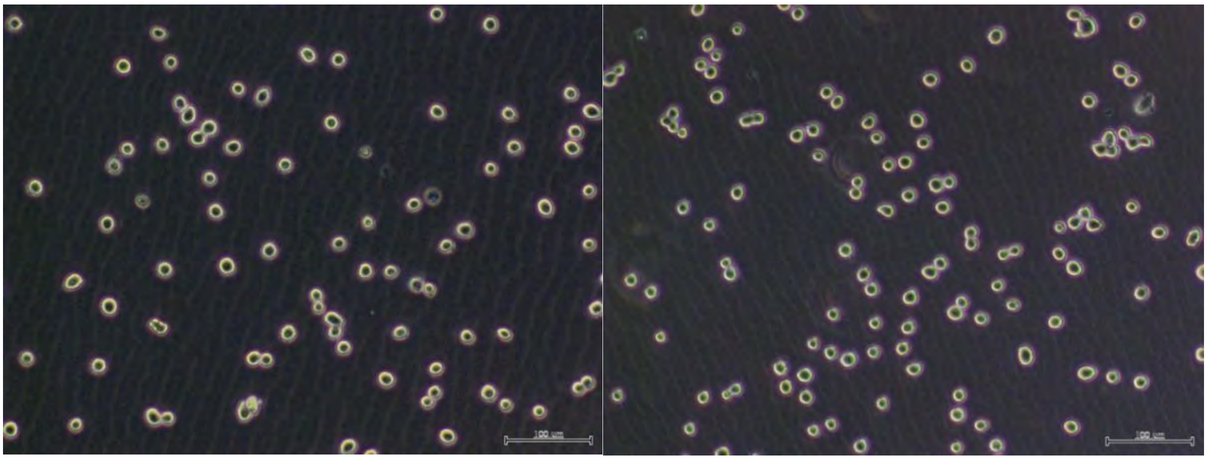


Figure S1.2 Osteoblasts – day 11 (before 1st passage).

S1.3 Osteoblasts at day 0 of co-culture.

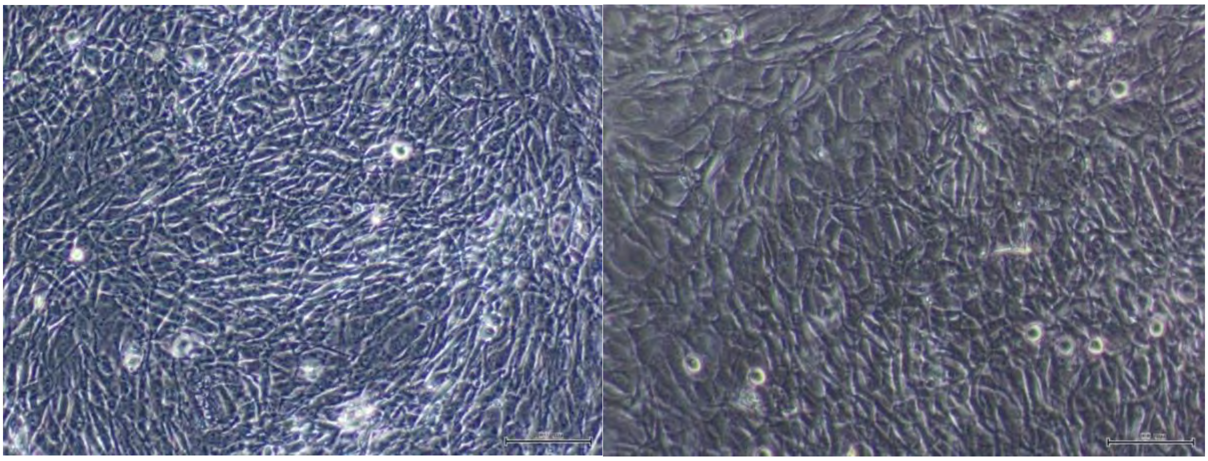


A B

Figure S1.3 Day 0 – co-culture.

A. Osteoblasts with normal media. B. Osteoblasts with conditioned HAMV media.

S1.4 Osteoblasts at day 7 of co-culture.

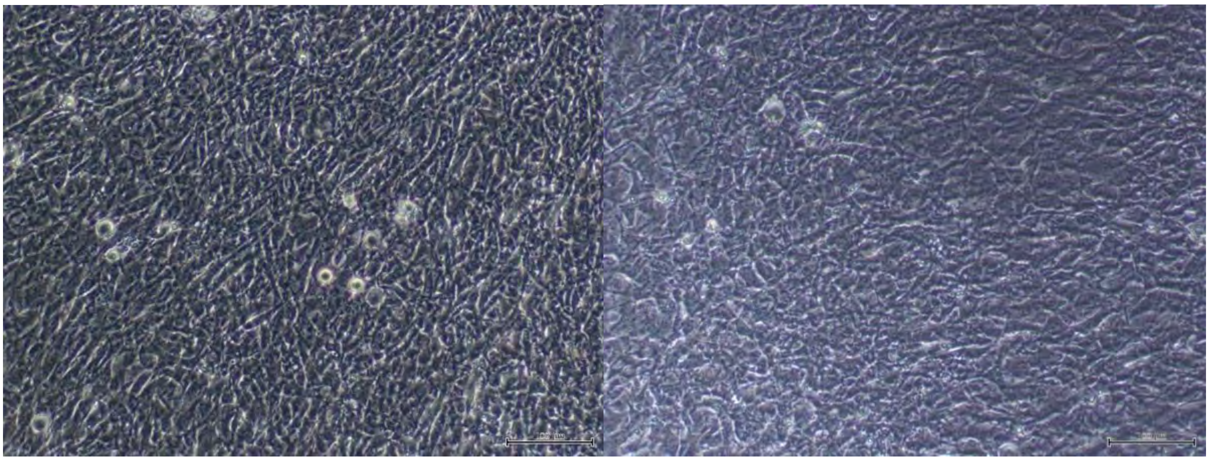


A B

Figure S1.4 Day 7 – co-culture.

A. Osteoblasts with normal media. B. Osteoblasts with conditioned HAMV media.

S1.5 Osteoblasts at day 18 of co-culture.



A B

Figure S1.5 Day 18 – co-culture.

A. Osteoblasts with normal media. B. Osteoblasts with conditioned HAMV media.

S2 Supplementary information for chapter five.

Sidak's multiple comparisons test

Table S2.1 Sidak's multiple comparisons test, test details.

D7 - D14	Predicted (LS) mean 1	Predicted (LS) mean 2	Predicted (LS) mean diff.	SE of diff.	N1	N2	t	DF
ALP	6.46	0.163	6.29	1.71	2	3	3.67	17.0
BMP-2	2.30	0.551	1.75	1.53	3	3	1.14	17.0
OCN	11.1	0.582	10.5	2.17	1	3	4.86	17.0
VEGF	0.902	0.345	0.557	1.53	3	3	0.364	17.0
Wnt-5a	2.11	0.793	1.31	1.53	3	3	0.857	17.0

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