# Towards the Development of Vascularized Constructs for Bone Repair

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*This thesis is dedicated to my mother, who had surgery on her maxilla to treat bone resorption, and to my father, who had a knee replacement; both during the course of this DPhil project. May we have better solutions to bone repair in the event that they require further surgery.*

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# Abstract

The development of a vasculature within a tissue-engineered construct is one of the largest hurdles to successful bone regeneration. This thesis investigates methods to increase vasculature of such transplanted constructs, based on *in viv*o transplant studies and *in vitro* analysis of cell behaviors. A syngeneic mouse model in immunocompetent mice was developed and analyzed for both osteogenesis and hematopoiesis. This study demonstrates that syngeneic bone marrow stromal cells (BMSCs) are not rejected by the host, provided the strain of mice is sufficiently inbred. Additionally, an effective protocol was developed for the isolation of endothelial cells (ECs) from the bone marrow of mice. Two different sets of materials for this study were analyzed, both collagen based, and the Gelfoam™ scaffold was found to possess advantages over synthesized collagen or collagen/hydroxyapatite composites, although only for mouse and not human bone transplantation.

In order to gain rapid and integrated vasculature formation within the transplant, attempts were made to increase both (*de novo*) vasculogenesis and angiogenesis (ingrowth) from the surrounding tissue. For the former, transplant studies were combined with *in vitro* osteogenic calcification studies. Direct co-culture of the BMSCs and ECs increased osteogenic calcification and was monitored by using both alizarin red S quantification and quantitative polymerase chain reaction. Angiogenesis (as assessed by cell migration) was studied by various motility and chemotaxis assays *in vitro*, as well as through use of a directed *in vivo* angiogenesis assay. Growth factors, particularly TGF-β1 and BMP-4, were found to increase cell movement in these systems. In conclusion, we show that although much work remains to be done in order to increase the vasculature in bone transplants, systematic combination of *in vivo* and *in vitro* assays can elucidate the nature behind this crucial process in this context.

# Abbreviations

2D = two dimensional

3D = three dimensional

αMEM = alpha minimal essential medium

αSMA = alpha smooth muscle actin

AAP = ascorbate-2-phosphate

Ang-1 = angiopoeitin-1

APC = allophycocyanin

ARCs = adventitial reticular cells

B-GP = β-glycerol phosphate

BME = basement membrane extract

BMP = bone morphogenetic protein

BSA = bovine serum albumin

BSP = bone sialoprotein

cDNA = complementary DNA

CFU-F = colony-forming unit fibroblast

CLS = capillary-like structures

CSDB = Craniofacial Skeletal Diseases Branch (at the NIDCR, NIH, DHHS)

DAB = diaminobenzidine

DAPI = 4',6-diamidino-2-phenylindole

Dex = dexamethasone

DHHS = Department of Health and Human Services

DHT = dehydrothermal treatment

DIVAA = directed *in vivo* angiogenesis assay

DNA = deoxyribonucleic acid

DPhil = Doctor of Philosophy

EC = endothelial cells

ECM = extracellular matrix

ECGS = endothelial cell growth supplement

EDC = 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide

ELISA = enzyme-linked immunosorbent assay

EtOH = ethanol

FACS =fluorescence-activated cell sorting

FBS = fetal bovine serum

FDA = Food and Drug Administration

FGF = fibroblast growth factor

FVB-N/J = Friend Leukemia Virus B (*Fv1b* allele) – National Institutes of Health/Jackson Laboratories

GAGs = glycosaminoglycans

GFP = green fluorescent protein

Glutamax = a commercially available solution of L-alanyl-L-glutamine

H&E = hematoxylin and eosin

$H_2O$ = water

HA = hydroxyapatite

hBMSCs = human bone marrow stromal cells

HIF = hypoxia-inducible transcription factor

HS = heparin sulphate

HUVECs = human umbilical vein endothelial cells

IGF = insulin-like growth factor

IgG = immunoglobulin G

MACS = magnetic-activated cell sorting

mBMSCs = mouse bone marrow stromal cells

mEC = mouse endothelial cells

MSC = mesenchymal stem cell

NIDCR = National Institute of Dental and Craniofacial Research (at the NIH, DHHS)

NIH = National Institutes of Health

NHS = N-hydroxysuccinimide

qPCR = quantitative PCR

PBS = phosphate buffered solution

PCR = polymerase chain reaction

PDGF = platelet-derived growth factor

PDGFR = PDGF receptor

PenStrep = a commercially available solution of penicillin and streptomycin

PFA = paraformaldehyde

PLA = poly-lactic acid

PTFE = polytetrofluoroethylene

PVC =polyvinyl chloride

RNA = ribonucleic acid

SEM = scanning electron microscope

siRNA = small interfering RNA

TCP = tricalcium phosphate

TGF-β = transforming growth factor beta

UV = ultra-violet

VEGF = vascular endothelial growth factor

VEGFR1 and VEGFR2 = VEGF receptors 1 and 2

# Chapter 1: Introduction

Musculoskeletal diseases are one of the leading causes of long-term pain and physical disability, affecting hundreds of millions of people worldwide [1]. There are many different causes of bone loss including, but not limited to, trauma, neoplasia, reconstructive surgery, congenital defects, and diseases such as osteoporosis and periodontal disease [2].

- There are over 57 million musculoskeletal injuries per year in the U.S. [3]
- About 15 million fractures occur per year in the United States and out of these, an estimated 5-10% fail to heal properly either due to non-union or delayed union [1, 4, 5]
- Only 3% of musculoskeletal injuries are taken to a hospital, yet they still comprise almost 1/3 of all hospitalizations for injury [3]
- About 80% of Americans adults have periodontal disease (to various extents), with severe cases leading to loss of cementum and surrounding bone tissue [1].
- Almost half of the adults between 45 and 65 years of age have moderate to severe periodontitis and associated alveolar bone loss, leading to the average loss of 6.5 teeth per individual if left untreated [6].
- Presently in the UK there are 50,000 hip replacement operations per year at a cost of £250 million [7]
- It is estimated that by the year 2050 there will be 6.3 million hip replacements per year worldwide, compared to 1.7 million in 1993 [7]
- 25-35% of large bone allografts fail 1-2 years after implantation due to propagation of microfractures in the grafted bone [8]

- Cost of musculoskeletal injuries is high not just because of direct costs, but also indirect loss of work hours [3]

In contrast to cartilage, bone is continuously undergoing regeneration and remodeling throughout the lifespan of vertebrates (including *Homo sapiens*). Osteoblasts are responsible for bone formation whereas osteoclasts are responsible for bone resorption. The balance of these two cell types leads to the turnover of bone and its capability to repair in response to trauma. Due to the turnover of bone, most bone lesions, e.g., fractures, can heal with the help of conventional therapy (primarily fixation through casting), especially in younger patients [9]. However there are select cases where other methods are called for, e.g., in the case of osteochondral defects, large traumatic defects, osseous congenital deformities [2], simple closed fractures, and instances of delayed healing, non-union, or segmental defects [10]. Thus, it is not an underestimate to say that therapies that can enhance bone regeneration and healing have the opportunity to better the lives of millions of people.

Tissue engineering is a scientific field encompassing biochemistry, molecular genetics, cellular biology, and material science, as well as biomechanical, chemical, and structural engineering. Two large categories of tissue engineering disciplines are in place: a) the formation of a new, functioning organ or tissue *ex vivo*, followed by its transplantation and integration with the existing system; and, b) the placement of all the component parts perceived as necessary for successful tissue engineering directly into the patient in order to allow development of the structure *in vivo* under physiological conditions and presumably to permit better integration of the developing bioengineered structure with the endogenous tissue [11]. In either case, the transplant should be a permanent solution for the damaged organ or tissue without the need for supplementary therapies [12]. The optimum tissue-engineered bone constructs for such circumstances as mentioned above would include the application of osteogenic growth

factors, osteoprogenitor cells, and a type of three-dimensional (3D) scaffold that will serve as a carrier for both factors and cells, as well as providing *in vivo* structural support for the regenerating bone.

## Background

### Non-Scaffold-Based Therapies

Currently, four main groups of surgical techniques used to treat large bone defects are: a) osteomy followed by bone distraction [13], b) various bone graft transplantations (auto-, allo-, and xeno-grafts, as well as synthetic bone fillers), c) the use of cell-occlusive barrier membranes (for periodontal disease) [2], and d) the use of various prosthetic devices (e.g., metallic hip and knee implants) [14]. It has long been recognized that only living cells that continue to synthesize matrix and remain metabolically active will be able to provide a viable tissue equivalent [15]; and also, studies have shown that artificial skeletal prostheses fail within 10-25 years in one out of two to three instances [16]. Therefore, neither c) nor d) above will be discussed, as we will focus solely on the regeneration of bone. The process of distraction osteogenesis avoids problems of graft integration and utilizes the natural regenerative capability of bone, but has a high complication rate and requires an extensive recovery period. Bone grafting is often used in instances of spinal fusion, revision of failed total hip arthroplasty or for the repair of skeletal defects following tumor resection or other trauma [17-20]. Grafting does indeed have a high "success" rate, but this success is also accompanied by frequent complications, e.g., non-unions and infections [21, 22]. An additional problem with the latter method is donor site morbidity, chronic pain, and complications of harvest procedures [15, 23-25]. Furthermore, although generally successful (and non-immunogenic [1]), such grafting of autologous bone is limited to the regenerative potential of the transplanted tissue, which might be compromised in some disease states [26]. Allografts require processing in order to make them more

acceptable to the host, but this processing, e.g., irradiation and/or long-term frozen storage, also leads to either rapid resorption or failure to remodel and incorporate with host bone, along with an increasing probability of graft fracture at a later date [27-32]. Although much research has been done, researchers have not yet been able to replace autografts as the predominant method of bone repair. Perhaps the most critical missing component in allografts in contrast to autografts is the lack of osteoprogenitor cells which can populate the new tissue and provide cues to surrounding tissues to help with bone repair [30, 33-36]. The lack of an optimal solution for bone segmental repair has led researchers to seek tissue-engineered systems that will overcome the limitations of the current options [37]. Researchers must strive to provide four main elements in any devised bone repair system [2, 14, 15, 38]:

- osteogenic progenitor cells
- osteoconductive matrices
- osteoinductive growth factors
- a sufficient blood supply

Each of these elements will be discussed in greater depth below.

## Scaffold-Based Therapies

The concept of tissue engineering was first popularized about 15 years ago [39]. Although initially focused on the regeneration of simple tissues e.g. the epidermis [40], the field has now moved on to encompass a wide range of organs and tissues throughout the body.

In theory, the combination of the correct adult stem cells (often expanded in culture), associated with the right biomaterials, will allow for the treatment of virtually any tissue injury [41]. Recently, much interest has been directed at the regeneration of cartilage, bone, and all associated soft tissues [42, 43]. Variations on the main triad of

components (numbers 1-3 above) have been tried singly and in combination; i.e., cells only, scaffolds only, growth factors only, and combinations of 2, 3, or more types of these components [38]. Thus, it is evident that the setup of a successful bone regeneration system along these lines will require knowledge of medicine, biochemistry, biology, materials science, and engineering [15].

Much research has been conducted on so-called bone marrow-derived "mesenchymal stem cells" (MSCs), more appropriately named bone marrow stromal cells (BMSCs), which show vast potential to differentiate into skeletal-type cells and additionally present the opportunity to be harvested from each individual patient, which should lead to higher immune tolerance of the transplant. However, due to limited bone marrow availability and the low numbers of cells within bone marrow, such cells must be expanded in *in vitro* culture and have been shown to have limited doubling potential prior to senescence, although they have indeed been shown to maintain multi-potency over an extended period of time [44]. Such cells have been shown to be stable [45] and are not suspected to suffer from growth deregulation *in vivo*. Moreover, the requirement of fetal bovine serum (FBS) for *in vitro* expansion is somewhat worrisome due to the xenogenic nature of this serum. However, the FDA has approved clinical studies in which FBS is used [15]. Additionally, research is also being conducted on possible alternatives to FBS, employing cocktails of stimulatory factors [46] or human platelet lysates [47].

The purpose of a scaffold is to provide a temporary support for bone regeneration, but should ultimately be resorbed and completely replaced by bone. The properties of the scaffold, both chemical and physical, are required for cell attachment, growth, and differentiation [12, 15, 48]. Although bone cell differentiation, proliferation, and production of extracellular matrix (ECM) proteins were originally studied in 2-dimensional (2D) monolayer culture, 3-dimensional (3D) culture was found to be more

realistic and a more accurate indicator of *in vivo* activity [14]. One of the major issues in choosing a scaffold is whether the scaffold is made of organic or inorganic material, as the choice of a scaffold's material can affect its functionality. One of the main steps forward in the field is the development of various composite materials that combine the optimum qualities of more than one material. Another major area of research is the modification of the scaffold shape (e.g., through solid freeform fabrication techniques [12, 49, 50], to produce more complex contours, such as channels which might increase the flow of fluid (either *in vivo* or in a bioreactor) through the scaffold. Such molding attempts both to increase vascularization (since *in vivo*, cells are no further than 20-200μm away from the nearest capillary) and to provide a temporary vasculature system in order to allow for easier flow of nutrients to, and waste from cells on the scaffold [15]. An extensive review of solid-freeform fabrication of scaffold materials can be found in Sachlos and Czernusza, 2003 [12]. Just one example of the intricacies of scaffold engineering and biology is the fact that cells produce more bone mineral when on scaffolds with non-homogenous pore size and shape, instead of a more controlled homogenous pore-structure [51].

When it comes to growth factors used in tissue engineering and regenerative therapy, bone formation is the well-recognized response of osteogenic cells to a diverse range of growth factors in specific spatial, temporal, and concentration-dependent combinations. Experiments have been conducted in which growth factor solutions or injectable extracellular matrix/self-assembling peptides [52] have been directly injected into a localized area. However, the use of a carrier, e.g. a 3D biomaterial sponge, was shown to be advantageous in order to localize the growth factor and slow down its delivery and clearance from the system [10]. Figure 1 [49] demonstrates the different factors which must be considered in order to design a scaffold for tissue regeneration.

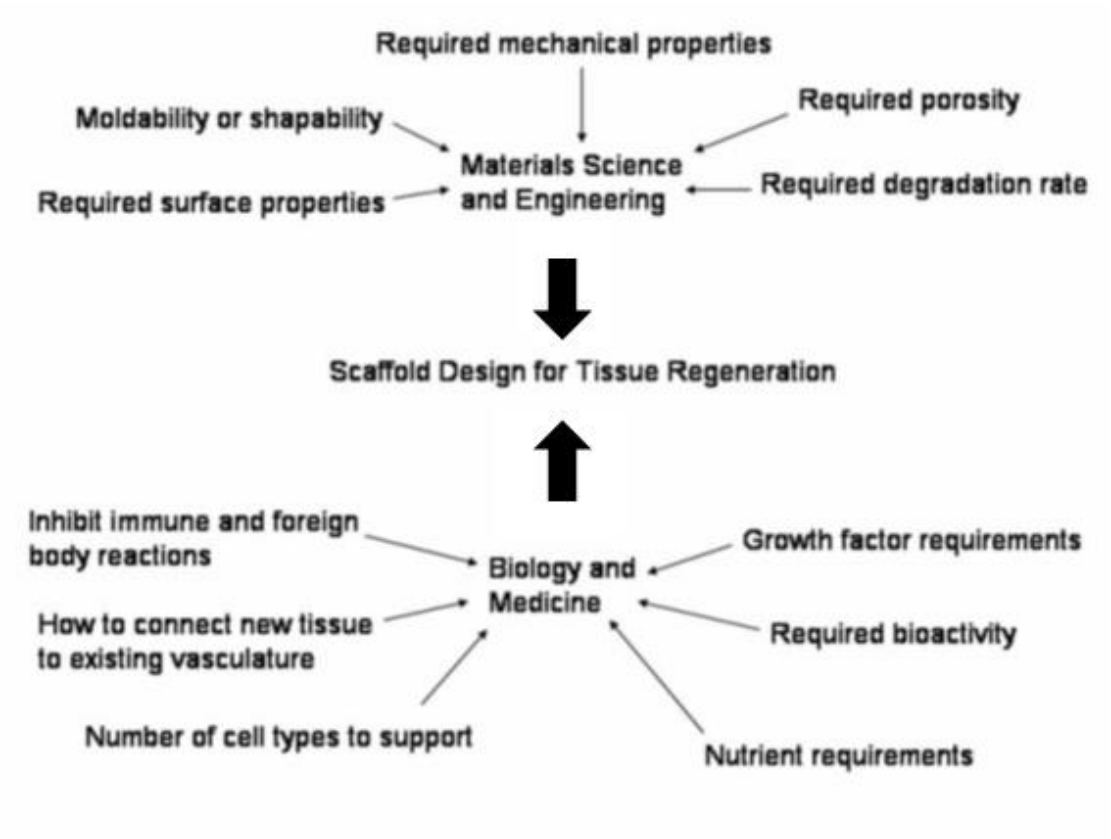



**Figure 1: Factors that should be considered when making a tissue-engineered construct.** Figure adapted from [49].

Animal models are used in tissue engineering in order to develop a basis on which therapeutic concepts can be formulated. They are indispensable for the primary experimentation to determine the basic science on which a regenerative system can be based. Various models have been used ranging from small (e.g., mouse) to large (e.g., dog and pig) [53-55]. In such models, many different factors are assessed, including, but not limited to, the potential for the scaffold system to create histologically normal new bone, to integrate with pre-existing tissues and vasculature, and to take into account the immune and toxicological response of the host to the transplant. The need for such animal models cannot be understated. *In vitro* experiments are prone to outside influence, with much more controlled environments, and with treatment protocols that do not resemble what physiologically occurs *in vivo*. Particularly in the case of bone, the reaction of the transplant to the mechanical stresses applied in a weight-bearing area is

a critical characterization study. Furthermore, transplantation of tissue engineering systems in humans first, without testing them in a living host at all, is considered unethical.

## Angiogenesis

### Importance of Angiogenesis

The human vasculature was once described by Aristotle:

***"the system of blood vessels in the body may be compared to those of water-courses which are constructed in gardens: they start from one source, or spring, and branch off into numerous channels, and then into still more, and so on progressively, so as to carry a supply to every part of the garden." [56]***

Bone healing and angiogenesis are synergistic and interactive processes [57], and both need to be considered for this project. The necessity for a vascular system for bone development, homeostasis, and repair was first documented in the eighteenth century [5]. However, bone research then focused predominantly on osteoblasts and other components of osteogenesis, until the late 20$^{th}$ century when vasculogenesis was again brought to the fore [58, 59]. The vasculature in any system is responsible for transporting nutrients, oxygen, and other soluble signaling factors to cells and the removal of waste and secreted signaling molecules away from them [49, 60]. Thus, it comes as no surprise that the development of angiogenesis is one of the earliest events in organogenesis [61]. The vasculature in bone is critical for organ development, homeostasis, and repair [62, 63] (with the exception of cartilage, which is avascular). Examples of the importance of angiogenesis can be found in pathology, where alterations in vascular state of bone tissue can lead to osteonecrosis [64], osteomyelitis [65], osteoporosis [66], and rickets [67]. Oftentimes, lack of vascular development is the cause of the poor bone healing in non-union fractures [5].

## Natural Process of Blood Vessel Formation

Blood vessels can be formed by two different, but parallel processes, angiogenesis and vasculogenesis. Angiogenesis is the sprouting of capillaries from pre-existing blood vessels [68], whereas vasculogenesis is the *in situ de novo* assembly of capillaries from previously undifferentiated endothelial cells (EC). There are 6 different steps to each process shown in Table 1 [49] and Figure 2 [49, 69]:

| Angiogenesis | Vasculogenesis |
|---|---|
| **Vasodilation** | ***In situ* differentiation of mesodermal cells into angioblasts and hemangioblasts** |
| **Basement membrane degradation** | **EC are generated from precursor cells (angioblasts)** |
| **Adherent cell migration** | **EC form the vessel primordium and aggregate to establish cell-cell contact but have no lumen** |
| **Formation of capillary lumen** | **A nascent endothelial tube is formed, composed of polarized EC** |
| **Synthesis of basement membrane** | **A primary vascular network is formed from an array of nascent endothelial tubes** |
| **Recruitment of pericytes and vascular smooth muscle cells** | **Pericytes and vascular smooth muscle cells are recruited** |

**Table 1: Differences in the processes of angiogenesis and vasculogenesis.** Table based on [49].

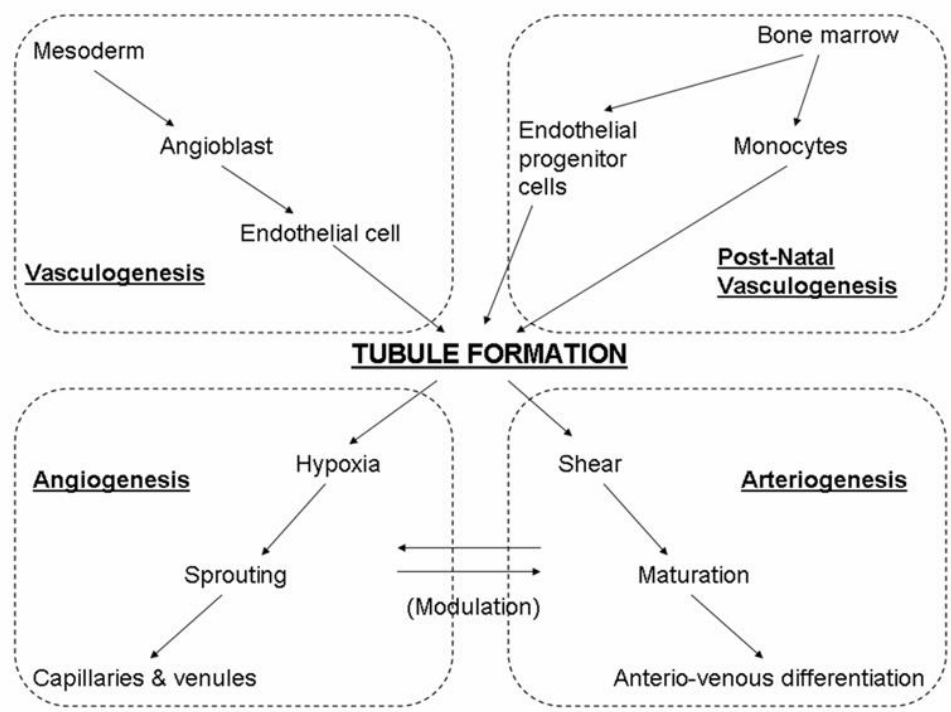



**Figure 2: The differentiation pathways for vasculogenesis, post-natal vasculogenesis, angiogenesis, and arteriogenesis.** Figure adapted from [49].

Adult vessel formation is thought to be the result of a combination of angiogenesis and vasculogenesis [70, 71], and stimulated by a mix of growth factors and ECM proteins. During embryonic development, vascular networks develop by *de novo* aggregation of angioblasts into a vascular plexus from which sprouting, bridging, and additional growth leads to establishment of the circulatory system [72]. Neoangiogenesis in an adult has been shown to follow a very similar pattern [73]. In order to grow and expand, blood vessels destabilize the surrounding tissue to allow new networks to form. However, this process also releases growth factors that have vital regulatory functions, which would otherwise be secreted in the matrix [56]. Some growth factors shown to be central to angiogenesis include angiopoetin-1 (Ang-1), fibroblast growth factor (FGF), hypoxia-inducible transcription factor (HIF), insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and members of the transforming growth factor-β (TGF-β) super-family, among others [5]. Endothelial tube formation is not only dependent on the balance of

soluble signaling factors, but also on the local ECM. Indeed, studies have shown that the simple addition of type I collagen to a monolayer ECM culture will induce the cells to form tubules, i.e., premature vessels [74, 75]. Therapies for vascular pathologies, either for over-active or deficient vasculature formation, are still in the infant stages, as it has become clear that the behavior of angiogenic moieties differs widely between organs and tissues and that what works in one organ might have negligible or undesirable effects in another [76].

Vascularization and bone formation are highly correlated processes, as quantified by Barou *et al.* [77], who measured angiogenesis through the use of India ink injections while simultaneously evaluating bone formation through dynamic histomorphometry. Vascularization of hypertrophic cartilage in long bones occurs in three separate stages and is closely linked with the process of endochondral bone formation [78]: in embryogenesis, blood vessels invade hypertrophic cartilage to form the primary spongiosa; at the end of puberty, capillaries invade the growth plates of the long bones; and during adulthood, angiogenesis can be switched on as necessary to support new bone growth in areas of trauma or remodeling [79]. Disruption of the balance of cartilage formation and vascular invasion can lead to growth plate thickening and a reduction in bone formation [80]. The main vascular systems in bone include the metaphyseal, epiphyseal, and periosteal networks [81].

Endothelial cells (ECs) provide the interface between blood and tissues by forming the blood vessel walls and acting as a semi-permeable membrane. The barrier these cells form mediates the passage of cells, fluid, and nutrients in and out of the blood system. ECs have tight junctions with each other as well as with pericytes [82, 83]. Under normal physiological conditions, vessels are stable, and not subject to leakage. However, many pathological systems exist (e.g., cancer) where the vasculature is destabilized, leading not only to angiogenesis but also to vascular leakage [82]. Injury

does damage not only to the bone and cartilage, but also to the bony vasculature, leading to the formation of a hematoma. This is followed by an immune response and the release of cytokines and growth factors that recruit bone forming cells to the area [14, 84]. Thus, spatial control of angiogenesis is particularly crucial because otherwise pathological effects can arise [85].

## Angiogenesis – Role in Tissue Engineering

The vascularization of implants has been one of the largest hurdles in the development of a successful bone regeneration system. Such vascularization is critical for the maintenance of cultured, seeded cells upon transplantation *in vivo* [37, 86, 87]. Two tissue-engineered systems that do not have this problem are skin and bladder [88] because they can, for all intents and purposes, rely on diffusion for transport of oxygen and nutrients through their essentially 2D conformation [89]. In many experimental 3D tissue-engineering systems, healthy tissue formation is well documented as being limited to the periphery of the implant, while interior necrosis results from inadequate perfusion [90-92]. Cells in any natural system are always close to capillaries, without which they will necrose (due to diffusion limitations of nutrients etc.). There are three main ways of getting scaffolds vascularized *in vivo*: a) the infiltration of exogenous cells into the scaffold once it has been implanted; b) the seeding of cells just prior to implantation, so that physiological conditions will stimulate these cells to undergo neovascularization; and c) the *in vitro* formation of vessels in order to attempt to speed up the process of vasculature formation upon implantation, provided that the scaffold vasculature interfaces correctly with pre-existing vasculature [49]. The problem with the "no cell" method (a) is that blood vessels require time to infiltrate the tissue. The "pre-vascularization" method (c) can give results *in vitro* that may appear to show highly developed vessels, but which may not be successful *in vivo*. Problems include integration of the artificially formed vessels with *in vivo* pre-existing vessels surrounding the transplant site as well as regression of budding vessels when placed *in*

*situ* [49]. The second approach (b) provides seeded cells in order to speed up the process of vascularization, but does not attempt to form vasculature prior to implantation. The challenge is to find the right balance of construct elements that will both allow ECs to invade the construct as well as to promote the differentiation of seeded cells into vessel-forming cells [93].

## Scaffold Materials

### Bone Development: A Brief Overview

Before proceeding further, a discussion of the nature of bone formation is in order. Of note, "bone formation" discussed here actually falls into three different categories: a) bone formation in the developing human skeleton, b) bone remodeling in an adult human skeleton, and c) bone formation in tissue engineered transplants.

The adult human skeleton comprises 206 different bones, which range from long bones in the limbs through short bones in wrists and ankles, flat bones in the skull and sternum, and irregularly shaped bones including vertebrae and the pelvis [9]. The bone organ consists of mineralized matrix with embedded cells (osteocytes) that encases bone marrow. From a developmental perspective, bone can be histologically sorted based on collagen fibril organization (observed through polarized light, as shown below).

**A)** **B)**

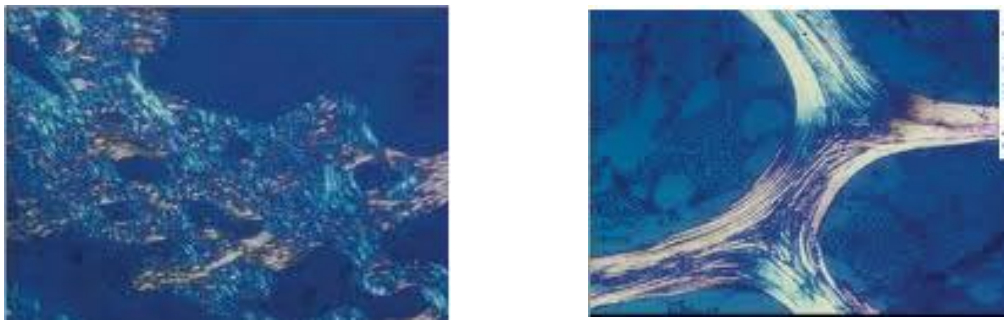


**Figure 3: A) Woven vs. B) lamellar bone.** This image shows decalcified sections stained with Weigert's hematoxylin and eosin and viewed with polarized light x 100. Modified from [94]

Woven bone generally occurs during embryonic development and in other cases of *de novo* bone formation such as wound healing (but also in disease states such as Paget's disease). Woven bone has a disorganized matrix structure. During development, it is remodeled into lamellar bone that has highly organized collagen fibril deposition. This organization of collagen in aligned layers placed at angles on top of each other allows for higher mechanical strength in lamellar bone. Mature bones can be placed in two categories, cortical (compact pattern) and cancellous (trabecular pattern) [95]. Cortical bone is primarily found on the exterior surface of bones, makes up the majority of the bone mass, is stiffer, and is made up of the osteon structures shown below in Figure 5. Cancellous bone, sometimes called spongy bone, is found toward the interior of the bone, and particularly at the ends of long bones and the interior of vertebrae. This bone type is highly vascular and is the site of hematopoiesis. The distribution patterns discussed are shown below in Figure 4.

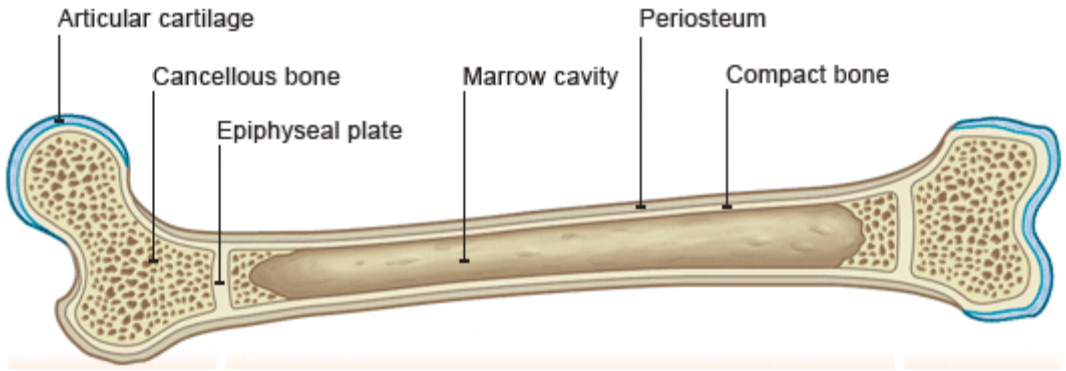



**Figure 4: Distribution of cortical (compact) and cancellous (trabecular bone) in a femur.** Figure modified from [96].

Regardless of location, bones have a distinct morphology and hierarchy that ranges from individual cells (osteocytes, osteoblasts, bone lining cells, bone marrow stromal cells, and osteoclasts, as well as adipocytes and cells of hematopoietic lineage) [97] through cellular connections, the complexity of the extracellular matrix (discussed more below), up to the various parts of bone including vascularization, resolving in the

whole structure as shown below in Figure 5 [9]. The degree of porosity and mineralization in bone has been shown to vary based upon location in the body. The cells involved in bone development are incredibly complex and their intricacies will be discussed, but one should likewise consider bone organ dynamics as a whole.

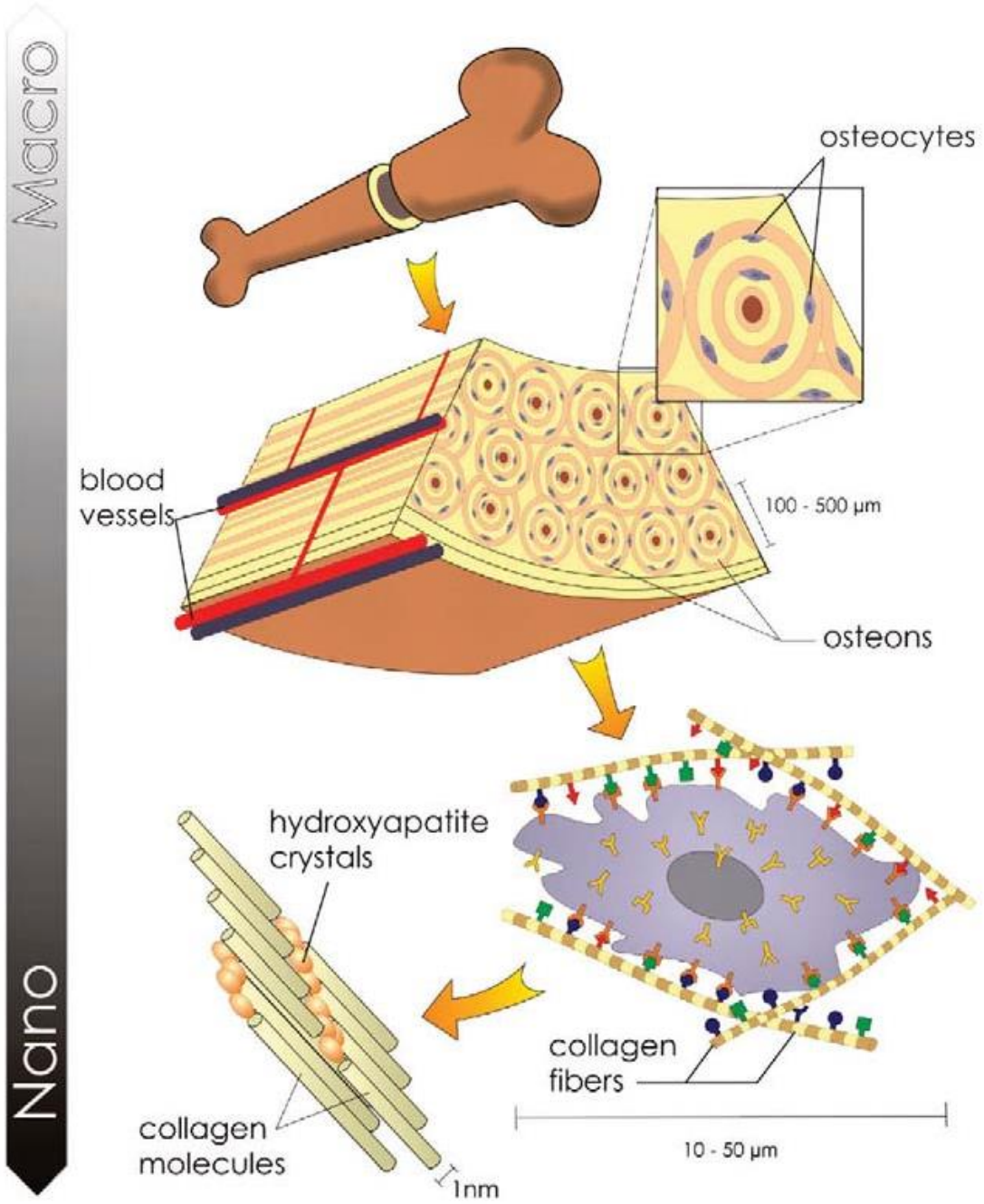



**Figure 5: The organization of natural bone.** Bone has a strong calcified outer layer (a), which comprises many cylindrical Haversian systems, or osteons (b). The resident cells are coated with a forest of cell membrane receptors that respond to specific binding sites (c) and the well-defined nanoarchitecture of the surrounding extracellular matrix. Figure adapted from [9].

The processes of bone repair and development, as well as the biochemical and mechanical properties of bone, depend on age, nutritional state, and activity (and disease state) [98]. Bone development can be viewed as having two (non-exclusive) phases. The first phase is the initial commitment phase – cells that will later become bone are localized according to specific hierarchy. The second phase is the differentiation phase – which is the induction of the cellular phenotypes necessary to produce bone [2].

Bone is derived from multi-potent fetal cells through two major routes: intra-membranous ossification and endochondral ossification. During endochondral ossification (esp. long bones, but also most other non-craniofacial bones), mesenchymal cells form bone through a spatially highly organized chondrocyte maturation process. During this process, cells move away from the epiphyses while they proliferate, condense, and differentiate into chondrocytes. These chondrocytes then hypertrophy, mineralize, and become vascularized through the invasion of blood vessels, followed by in-growth of osteoblasts from the diaphyseal side of this cartilaginous structure [99]. Histologically, this is observed as a series of zones.

During intra-membranous ossification (many craniofacial bones and the clavicle), there is no apparent cartilage intermediate. Instead, multipotent mesenchymal cells group into dense aggregates (nodules) and differentiate into osteoprogenitor cells. These nodules then become osteoblastic, secrete extracellular matrix, vascularize and mineralize[1]. The differences in gene expression between endochondral and intramembranous ossification have been characterized [100].

A third, less well known process of bone development is MT-1 (membrane type-1) MMP-(matrix metalloproteinase) dependent remodeling [101]. MT-1 MMP-dependent remodeling occurs in specific skeletal areas, including calvarial bones,

mandible bones, and is related to periosteal bone formation at Ranvier's groove in long bones. Unlike endochondral ossification, unmineralized cartilage is remodeled through MT-1 MMP-dependent ECM degradation, combined with coordinated chondrocyte apoptosis. As unmineralized cartilage disappears, bone appears [101].

After puberty, human growth plates close in a process known as epiphyseal fusion (note that this does not happen in mice). Growth plates are responsible for driving longitudinal growth via endochondral ossification. The growth plate itself is a highly ordered arrangement of chondrogenic tissue, which becomes ossified at one end through creeping substitution, while continuing to produce more chondrogenic tissue at the other through the maturation of immature chondrocytes [102]. Although there are many hypotheses about how epiphyseal fusion occurs [102], there is a distinction to be made between bone remodeling and healing and bone development.

Bone in adults is constantly being broken down and regenerated, one of the few "organs" in the body with a relatively high rate of turnover [103]. At any given time, up to about 7% of the natural bone surface is undergoing bone remodeling [104]. Bone is remodeled in order to modulate circulating calcium and also to repair microfractures. This remodeling process is driven by a complex set of cells discussed below in the Cells section. Essentially, bone is degraded by osteoclasts, and osteoblasts are cells that are actively depositing new bone. A high level of regulation keeps the whole system in balance in order to prevent either insufficient or over-exuberant bone degradation or formation.

Tissue engineering differs from bone remodeling in many respects, not least in that there is no existing bone to remodel. Instead, scientists provide components needed to stimulate an adult version of original skeletal development. Eventually, it is hoped that the bone formed will be remodeled by natural processes, in order to fully integrate with the existing surrounding bone. Overall, studies have shown that there are

three main requirements for the development of bone, all of which must be met by tissue engineering constructs [105]:

1) the presence of collagen

2) the availability of phosphate (and calcium)

3) removal of pyrophosphate and absence of other inhibitors of mineralization

## The Use of Synthetic vs. Natural Materials in Tissue Engineering

Although not studied in this research project, the use of autografts, allografts, and decellularized tissues should be briefly discussed. Autografts, as mentioned above, are currently the gold standard in terms of bone replacement [1]. However, bone autografts are limited by availability (traditionally from the iliac crest) as well as other complications [15, 24, 25]. Autografts present several advantages over other solutions – inherent mechanical stress, lack of *ex vivo* culture, and minimal immune response risk. Allografts possess many of the same advantages as autografts and are the most common solution in bone transplantation [27, 28]; however, they do present several additional cons. There is a higher risk of immune response, even though donor and host are MHC-matched. Also, for many autografts, tissue harvesting and transplantation occur in the same surgery, whereas in the case of allografts, tissue often is frozen or freeze-dried in tissue banks. This step can decrease tissue viability, although it is a necessary step for long-term preservation of tissue [29-31]. Decellularized tissue is material that has been highly processed to remove all cells. Human tissue that has been processed in this way can be reseeded with the patient's own cells in order to provide a pseudo-autogenic material [106]. Xenogenic material is less common, and requires additional processing over human material, in order to remove any immunogenic foreign material [107]. In either instance, in the case of bone, such material must be also demineralized, in order to remove osteocytes that have been embedded within the calcified matrix. Based upon this consideration, decellularized, demineralized bone will therefore not provide the

same mechanical strength as a graft. Nevertheless, such material does provide a scaffold made up of a completely natural mix of biological polymers that has not yet been replicated in the laboratory.

One of the main decisions facing a researcher aiming towards designing 3D osteogenic tissue scaffolds from scratch (instead of utilizing one of the options above) is whether to use a synthetic or a natural material since each presents advantages and disadvantages [108]. The ideal material would be [41, 49]:

1) non-immunogenic,
2) non-toxic (both at the time of implantation and throughout the process of degradation),
3) biocompatible,
4) biodegradable, having the proper mechanical and chemical stability to provide structure for osteogenesis, but make room for new bone formation on the correct timescale,
5) provide a possible mechanism of integration with pre-existing bony and cartilaginous matrix around the lesion site,
6) and easy to manufacture (for a reasonable cost) .

In general, synthetic materials have a more easily controlled pore morphology and can provide better mechanical properties, as well as having unlimited availability [49]. However, synthetic materials are not in and of themselves bioactive, can be immunogenic, and can produce toxic by-products upon degradation [12]. It should be noted that historically scaffolds were viewed as inert and merely physical support, but it is now understood that scaffolds play a bioactive role by directly binding to cells and through binding to growth factors [39, 109, 110]. Examples of synthetic material

currently in use in tissue engineering constructs include polylactic acid, polyglycolic acid (either in homo- or heteropolymers), polyprolactone, and polyvinyl alcohol hydrogels [5, 41], and various techniques have been utilized to derive the desired morphology of these scaffolds [111].

Natural materials form more heterogeneous porosity both on the macro and micro level, (the role of porosity is discussed more below), and are bioactive, interacting with cells, growth factors, and other signaling molecules. However, natural materials are limited by their availability, the necessity to ensure adequate purification in order to prevent transmission of infection, and their weaker mechanical properties. Because of their weaker mechanics, natural polymers are often modified by crosslinking or used in conjunction with non-natural materials; modifications that could lead to a less biocompatible material [112]. As discussed above, some of the more common natural materials which have been studied to date include: collagen, fibrin, and hyaluronic acid (all of which are members of the natural ECM in bone) [41] as well as silk fibroin [98].

Although bioceramics such as hydroxyapatite (HA) and tricalcium phosphate (TCP) are not polymers, they can be used either by themselves, in combination with each other, or combined with natural or synthetic polymers [5, 113, 114]. Bone can be simplified to "a polymer matrix reinforced with ceramic particles" [9, 12] where collagen and HA are present in a 30: 70 dry weight ratio. HA and/or TCP has often been used in the literature in order to promote the osteoconductive properties of natural polymers, or to confer bioactivity to synthetic polymers [23, 115, 116]. A higher TCP: HA ratio increases resorbability of the scaffold, whereas a lower ratio results in a more degradation-resistant scaffold [41, 117]. The problem with using bioceramics by themselves instead of in a composite material is that they are inherently brittle and cannot match the mechanical properties of bone [12]. The idea behind the formation of composite materials is to gain from the advantages of each composite material [49], as

demonstrated by the combination of the mechanical strength of an inorganic material (either synthetic or bioceramic) with the bioactivity derived from a natural polymer [9].

## Collagen

The collagen family accounts for about 30% of all body protein in vertebrates, present in different proportions in different types of tissues, e.g., 90% of ECM protein in tendon and bone and over 50% in skin [118]. In total, there are over 200 different types of ECM proteins, many of which are present in trace amounts [9]. Although collagen has been used for various clinical applications for a long time, researchers in the 1970s and 1980s started to consider collagen as a material with vast applications in biomaterials and connective tissue research. The term "collagen" is generally applied to the fibrillar and triple-helical, and aggregate, forms of the collagen, whereas the term "gelatin" is used for denatured/non-helical collagen [119]. In addition to providing mechanical strength and elasticity [120], collagen also acts as an important growth factor-binding site in the extracellular matrix, helping to localize and orient these regulatory molecules, including collagen-heparan interactions [48]. Each member of the collagen family contains regions of three left-handed helix chains that wrap around each other for a unique triple helix formation. Triple helices then bind together with specific configurations to form collagen fibrils as shown below [119].

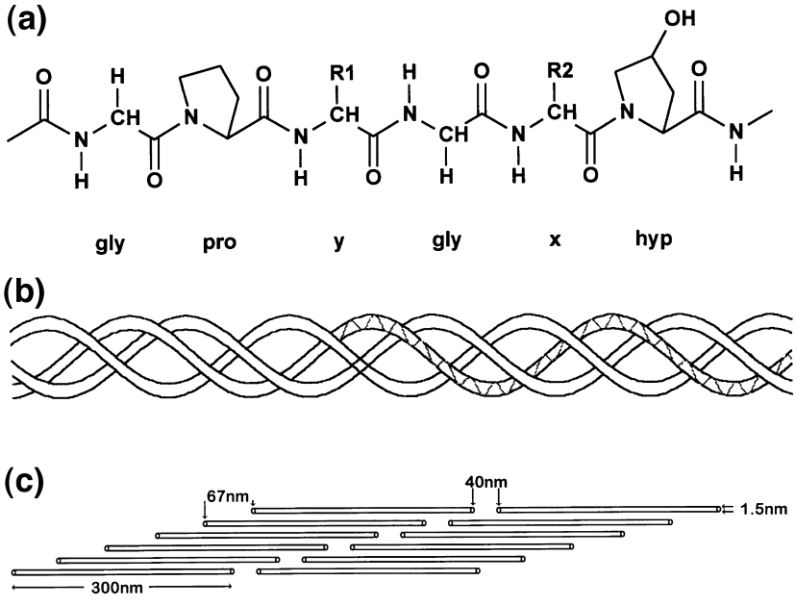



**Figure 6: The structure of collagen**, from single polypeptide strand (a) to triple-helix structure (b), and primary fibril structure (c). Figure adapted from [119].

Type I collagen is the collagen predominantly found in higher order animals in areas where extreme force can be applied; e.g., skin, tendon, and bone. It is composed of two identical polypeptides, the α1 subunit, and one chain of the α2 subunit, which has a different amino acid composition. Although rare, sometimes a homotrimer of α1 subunits is formed. The regional specificity and chemical characterization of some other collagen types is shown [119, 121].

| Collagen Type | Chain Composition | Tissue Distribution | Classification |
|---|---|---|---|
| **I** | **$(\alpha1(I))_2\alpha2(I)$, trimer $(\alpha1(I))_3$** | **Skin, tendon, bone, cornea, dentin, fibrocartilage, large vessels, intestine, uterus, dermis** | **Fibril-forming** |
| **II** | **$(\alpha1(II))_3$** | **Hyaline cartilage, vitreous humor, nucleus pulposus, notochord** | **Fibril-forming** |

| Collagen Type | Chain Composition | Tissue Distribution | Classification |
|---|---|---|---|
| III | $(\alpha(III))_3$ | Large vessels, uterine wall, dermis, intestine, heart valve, gingiva (usually coexists with type I except in bone, tendon, cornea) | Fibril-forming |
| IV | $(\alpha1(IV))_2\alpha2(IV)$ | Basement membranes | Network-forming |
| V | $\alpha1(V)\alpha2(V)\alpha3(V)$ or $(\alpha1(V))_2\alpha2(V)$ or $(\alpha1(V))_3$ | Cornea, placental membranes, bone, large vessels, hyaline cartilage, gingiva | Fibril-forming |
| VI | $\alpha1(VI)\alpha2(VI)\alpha3(VI)$ | Descemet's membrane, skin, nucleus pulposus, heart muscle | Beaded-filament-forming |
| VII | $(\alpha1(VI)\alpha2(VI)\alpha3(VI)$ | Skin, placenta, lung, cartilage, cornea | Anchoring fibrils |
| VIII | $\alpha1(VIII)\alpha2(VIII)$ chain organization of helix unknown | Produced by endothelial cells, Descemet's membrane | Network-forming |
| IX | $\alpha1(IX)\alpha2(IX)\alpha3(IX)$ | Cartilage, co-distributes with type II | FACIT |
| X | $(\alpha1(X))_3$ | Hypertrophic and mineralized cartilage | Network-forming |
| XI | $1\alpha2\alpha3\alpha_1$ or $\alpha1(XI)\alpha2(XI)\alpha3(XI)$ | Cartilage, intervertebral disc, vitreous humor, codistributes with collagen II | Fibril-forming |
| XII | $(\alpha1(XII))_3$ | Found with Collagen I | Anchoring fibrils |
| XIII | Unknown | Fetal skin, bone, intestinal mucosa, neuromuscular junctions | Transmembrane |

**Table 2: Chain composition and distribution within the body** for the first 13 (of 28 or more) types of collagen. Modified from [119, 121].

The immunogenicity of collagen is a subject that must be carefully examined if it is to be used as a component of an implant in human patients. Non-human-derived collagen displays excellent biocompatibility with low toxicity and minimal immunogenic reactions, features thought to stem from the homology between collagen in different species and the low content of aromatic amino acids [122]. The use of a pepsin-solubilized bovine dermal collagen has been extensively studied for immunological response [123, 124] and was shown to induce anti-implant antibodies in 3% of patients after a single injection and an additional 2% after a second injection. Thus, collagen can be considered only mildly antigenic [125] and hence a suitable biomaterial for tissue engineering.

The amino acid sequences of collagen chains are highly conserved between vertebrate species [122] and demonstrate a distinct pattern in order to allow for triple-helix packing. Every third amino acid in collagen is a glycine (the amino acid with the smallest side group, a hydrogen atom). In the sequence Gly-X-Y, proline is found in about 35% at the X position, or at the Y position as hydroxyproline, a rare form of the amino acid derived from post-translational modification. Another unusual amino acid found in collagen is hydroxylysine (also formed through post-translational modification) [119]. The actions of hydroxyproline and hydroxylysine help stabilize the triple helix through the formation of hydrogen bonds that limit rotation and lock the chains in place relative to each other. In addition, hydroxylysine allows for the attachment of carbohydrates (either a single galactose unit, or a disaccharide of galactose and glucose) which make up less than 1% of the total glycoprotein weight [118]. As shown in Figure 6 and Table 2 above, the individual collagen polypeptides assemble into a triple-chain known as the “tropohelix”, which has an average molecular weight of 300kDa, a length of 300nm and a diameter of 1.5nm [126]. Upon formation of tropocollagen, telopeptides (non-helical) sections are left at the amino and carboxyl ends, and are cleaved off prior

to fibril formation. Microfibrils of four to eight collagen triple-helices are formed, and then aggregate into fibrils that can range from 10-500 nm in diameter depending on tissue type and stage of development. Within each fibril, the triple-helix units are staggered by 67 nm and have an additional 40 nm gap between succeeding molecules. These fibrils then can organize into fibers that are even larger [126].

The systematic packing system of collagen gives strength to the collagen fibers that are heavily reinforced by molecular crosslinking within each triple helix and between the triple helices throughout all levels of organization, giving collagen unique resilience [126]. The degree of crosslinking varies depending on age and stress [127] and is a factor to be considered when designing a collagen-based 3D tissue scaffold. The natural method of crosslinking occurs through an enzymatic reaction and a series of condensations as shown below [119].

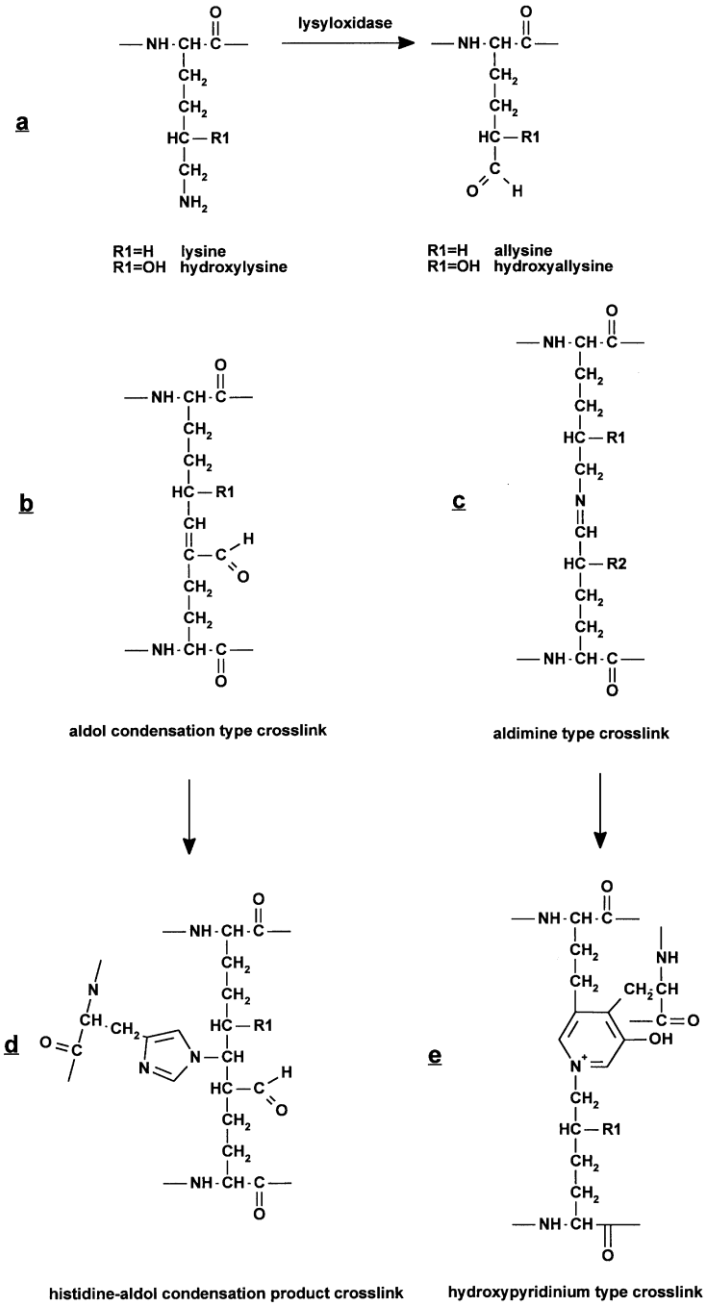



**Figure 7: The natural crosslinking process of collagen.** Figure adapted from [119].

Crosslinking allows the fibrils not only to withstand mechanical stress, but also serves to protect from chemical degradation. Collagen is not degraded by non-collagen-specific proteases. Collagenases are zinc-containing matrix metalloproteinases which cleave about three-quarters of the distance away from the N-terminus of the protein [126] and degrade the fibril from the exterior towards the interior (dense packing of the

fibril means it is impossible for anything to penetrate the interior). Once the collagen is broken down to a cleaved triple-helix, the triple-helix is then broken down by gelatinases and non-specific proteases.

Various methods have been used to stimulate the crosslinking of collagen *ex vivo* and fall into two categories: physical and chemical. The latter methods include: chromium tanning, with the chromium serving as a base for the formation of additional cross-collagen ionic bonds; treatment with formaldehyde or glutaraldehyde, which result in the formation of covalent bonds; and the use of various carbodiimides, especially 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), which have the advantage over aldehydes in that they are not actually present in between the two collagen molecules crosslinking, but rather stimulate direct carboxylic acid-amino group crosslinking [128], to give a few examples [119]. A comparison of glutaraldehyde, acyl azide, hexamethylenediisocyanate, and EDC crosslinking found that EDC cross-linking resulted in the most promising scaffold with a low rate of degradation, low immunogenic response, and the stimulation of new tissue regeneration [129]. One of the main drawbacks with chemical crosslinking is the stimulation of an immune response. Various physical methods have been suggested to get around the immunogenicity of chemical crosslinking while still providing strong scaffolds. Physical methods include, but are not limited to: exposure to UV irradiation, dehydrothermal treatment (DHT), and exposure to dry heat. These treatments have been shown to partially denature the collagen, though this denaturation was shown to be insignificant at temperatures below 120°C [130].

The administration of collagen as a three-dimensional scaffold into a patient presents a unique situation and the success of this application is reliant on the different types of cells invading the scaffold (seeding of cells on the scaffold will be discussed in another section) as well as the physical characteristics of the scaffold, particularly

porosity (also discussed below). Studies have shown that the implantation of collagen scaffolds that have not been treated to have additional crosslinking degraded *in vivo* within 2-7 weeks [125], although this can vary depending on the species of seeded cells and transplant host. Degradation occurs both through the action of extracellular proteases and through the lysosomal breakdown of partially cleaved fibers that have been phagocytosed [119].

Thus, we can see that while collagen, like all other polymers, both synthetic and natural, presents its own set of complications for use in tissue engineering, it is uniquely suitable for the base material from which to fashion a 3D scaffold for transplantation. Collagen can be summarized to have four main advantages over other materials available [119]:

1) good biocompatibility and well characterized low antigenicity,
2) degradation into well tolerated physiological compounds,
3) processing on aqueous base,
4) and enhancement of cellular penetration and wound repair.

### Hydroxyapatite

The use of ceramics by themselves has not been met with much success as they are inherently brittle and unable to match the mechanical characteristics of bone. Hydroxyapatite (HA), with the structure $Ca_{10}(PO_4)_6(OH)_2$ in which the hydroxyl group is often substituted with carbonate, is the predominant biomineral found in bones and teeth and has been used in many different biomaterial composites in order to increase bioactivity. Research has particularly focused on the use of HA incorporated into collagen scaffolds through various methods [131-136] and demonstrated to have osteogenic potential. HA has also been hypothesized to work well with synthetic polymer scaffolds, mitigating the acid released upon their degradation *in vivo*, which

would otherwise be damaging to the surrounding tissue [137-139]. There are many possible combinations of ceramics and polymers, both synthetic and natural, as shown in Figure 8 [140]. Research into the interactions between ceramics and human bone marrow stromal cells (hBMSCs) has also been attempted [141].

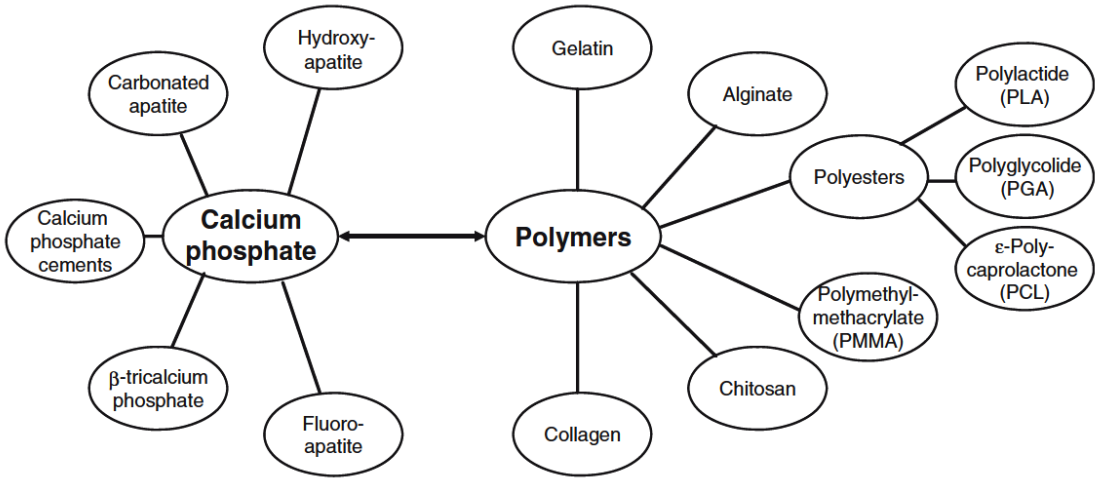



**Figure 8: Different combinations of calcium phosphates and polymers in tissue engineering scaffolds.** Figure adapted from [140].

The other ceramic which has been widely studied for tissue engineering purposes is tricalcium phosphate (TCP), with the structure $Ca_3(PO_4)_2$, which, like HA, is too brittle to be used on its own but has potential for use in composite materials [142, 143], including in conjunction with HA when seeded with osteogenic cell populations [144]. Studies have shown that HA and TCP together interact and are capable of supporting new bone formation at around 8 weeks when inserted into cylindrical bone defects in rabbits [145]. Additionally, studies show that human osteogenic cells seeded on HA/TCP scaffolds *in vitro* express osteoblastic proteins at higher levels over the first 10 days [146].

Although HA is not a good substrate for cell attachment by itself, it binds to proteins in the extracellular matrix (preferentially through the carboxyl groups on

aspartate and glutamate) that promote cell attachment [11, 147]. HA to collagen in a 30: 70 ratio by weight was selected in this study since that is the natural ratio in bone. Previous studies with collagen/HA composite scaffolds indicate that osteogenic cells can maintain viable osteogenic capability on these scaffolds at least over a 4 week period [148].

### Gelfoam™

Gelfoam™ is a product commercially available from Pharmacia and UpJohn (a subsidiary of Pfizer). The product has been FDA-approved for use as a hemostatic device during surgery. The sponge has been shown to be completely resorbable in small amounts on the order of days to weeks depending on the location of sponge [149]. The sponge has an irregular macropore structure [150], which will be discussed below. Gelfoam™ does not fray when cut [149] and has edges with the same porosity and properties as an inner cross-section (discussed in the Chapter 3). Gelatin sponges have had their absorption [151] and hemostatic [152] properties well described. One of the earliest studies on Gelfoam™ [151] found that the material was well suited for improving the healing of experimental liver resections. Liu *et al*. [150] performed studies comparing Gelfoam™ with gelatin scaffolds with similar properties. Studies of VEGF immobilization and the measurement of endothelial cell (EC) penetration into the scaffold during *in vitro* culture have shown promising results [153]. McCarty [154] demonstrated seeding of cells onto a Gelfoam™ sponge and use of a fibrin clot for transplantation into small growth plate defects in lambs. Other *in vivo* studies include Lee [155], which found that cell seeded Gelfoam™ as a covering for a bone allograft could greatly increase healing, as demonstrated by histology, and increased levels of relevant growth factors (measured by RT-PCR). Clarke [156] took a similar approach and used βTCP-loaded Gelfoam™ as a coating for a steel pin. Angiogenesis in Gelfoam™ has been previously observed through transplantation in mouse expressing GFP (green

fluorescent protein) and the imaging of GFP-labeled blood vessel growth into the scaffold after 7 days [157].

## Importance of Porosity and Other Physical Properties

Porosity is defined as the percentage of void space in a solid [158]. It is crucial for the purposes of tissue engineering that the implant material be porous. Without porosity, cells cannot penetrate into the construct and it does not form new tissue. Many studies have demonstrated this need for porosity with HA [159], and the use of various porous titanium treatments [160-162]. Since porosity has been demonstrated to be crucial for osteogenesis, the question is what degree of porosity, and what pore size, is necessary. The dependence of scaffold success on porosity and pore size has been shown to vary depending on the cells seeded and outcome desired. Different materials present challenges in terms of their construction leading to different pore size and porosity characteristics. For example, electrospun scaffolds, among others, are problematic because their pore size is relatively small (compared to the diameter of most cells), and salt-leached scaffolds often suffer from collapse of the 3D structure upon removal of the salt [163].

In addition to macroporosity, microporosity (on the scale of 100 μm or less) is necessary because it adds surface area and increases surface roughness [164-166]. Such microporosity is thought to lead to better adsorption of osteogenic factors and stimulate the attachment, proliferation, and differentiation of osteogenic cells [166]. Scaffolds which contained uniform pore sizes were found to promote discontinuous growth of bone islands, whereas scaffolds that contained a variety of pore sizes induced bone growth from the periphery of the scaffold towards the center (via creeping substitution) [167]. Changing the roughness of the scaffold has also been tested, particularly in PLA scaffolds, which are challenging in that they are normally hydrophobic [168].

The materials in a scaffold and its porosity will affect its mechanical properties [5, 98]. For making a scaffold out of collagen or collagen/HA suspension through freezing, the pore size and porosity of the scaffold is directly related to the freezing temperature as shown below [169].

$$|^{o}\mathrm{C}| \propto \dot{N} \propto P \propto \frac{1}{d} \propto \frac{1}{w}$$

**Figure 9: Equation explaining the correlation of processing factors with pore size and collagen wall thickness in scaffolds.** °C is the temperature at which the solution is frozen – i.e. the extent of undercooling, and is related to the number of crystal nucleation events that occur (N). At lower temperature with more nucleation events and slower diffusion of these nuclei due to undercooling, a higher number of pores (P) are formed in a fixed amount of space, each with a smaller diameter (d) and the collagen is pressed into thinner walls (wall width = w). Figure adapted from [169].

Given that the same amount of collagen goes into each scaffold that are all the same size, regardless of the porosity, the larger the pores are, the fewer pores there will be and the thicker each wall. Hence, we see that porosity, not just the material from which the scaffold is made, will play a role in the mechanical properties of the scaffold. The balance between mechanical strength and porosity is a key factor to osteogenesis and the success of a tissue engineering construct [170]. One major way to alter the mechanical properties of 3D scaffolds of collagen is the crosslinking of the collagen either through physical or chemical methods. These methods, discussed above, provide a way of strengthening the structure without affecting its porosity. In addition, cells have been shown to demonstrate a behavioral dependence on matrix stiffness. Durotaxis is the process whereby cells spread better on stiffer substrates and spreading has also been shown to be related to the density of signaling factors and ECM ligands [171]. Numerous studies have been conducted on whether changing the alignment of materials can enhance cell growth into the scaffold, but have generally had inconclusive results [172-176].

# Cells

## Cells in Tissue Engineering: Concepts

### *Level of Understanding in the Field*

Tissue engineering research has predominantly focused on scaffold and bioreactor development, also including the mechanical stimulation of constructs to induce tissue development [177], and less so on the cells used in tissue engineering constructs [178]. The cells used in the experiments described below include human and mouse bone marrow stromal cells (hBMSCs and mBMSCs) and human umbilical vein endothelial cells (HUVECs). hBMSCs have, of recent, often been referred to as "human mesenchymal stem cells," a misleading term as will be discussed below. As mentioned above, vascularization of the scaffold is integral to its success as a tissue-engineering construct. In the absence of seeded cells, the construct is reliant on endogenous cells at the implantation site to migrate into the scaffold to populate it, vascularize it, and form appropriate tissues. The traditional paradigm, and the one presented in this thesis, involves the seeding of cells onto the scaffold. The seeded scaffolds are then cultured *in vitro* (for possible future implantation) or directly transplanted into the test host. This approach should, in theory, work by allowing the *in situ* formation of blood vessels that would then connect to the pre-existing vasculature, allowing for delivery of nutrients to the cells within the scaffolds at a rate faster than the endogenous vasculature could permeate the scaffold [98]. Cells from many different adult tissue types have been investigated for their possible use in osteogenic tissue engineering applications. However, many of these sources do not stand up to intense scrutiny. Indeed, the very foundations of many published experiments result from gross misunderstanding of osteoprogenitor cell biology that is pervasive throughout the field of regenerative medicine. To demonstrate this, the osteogenic and hematopoietic stem cell microenvironment will be explained briefly, along with the misconceptions in the field

about the term "mesenchymal stem cell", and then delve into the complex world of osteogenic differentiation. Only after this will the cells used in this project be discussed: BMSCs and ECs. A summary of the terms used for this section is provided below in Table 3 for reference.

| Term | Abbreviation | Definition |
|---|---|---|
| **Adventitial reticular cell** | **ARC** | **ARCs are pericytes with the ability to self-renew and also produce cells of osteogenic lineage.** |
| **Bone marrow** | **BM** | **The tissue found in the interior of bones that contains the hematopoietic and osteogenic progenitors. Also the site of bone turnover.** |
| **Bone marrow stromal cell** | **BMSC** | **A term specific to adherent cells in culture which are derived from a full bone marrow flush** |
| **Colony forming unit-fibroblast** | **CFU-F** | **The in vitro equivalent to ARCs, a subset of BMSCs with the ability to generate colonies** |
| **Endosteal surface** | | **The area of tissue that lines the bone surface** |
| **Mesenchymal stem cell** | **MSC** | **A stem cell derived from mesoderm capable of self-renewal and also capable of generating all cells of mesenchymal origin** |
| **Osteoblast** | | **Cells which are actively depositing bone at sites of bone turnover** |
| **Osteocyte** | | **Cells which have progressed down the osteogenic lineage and are embedded in calcified bone** |
| **Pericyte** | | **A cell lining the microvasculature. In the bone some pericytes are ARCs.** |
| **Sinusoid** | | **A specialized microvasculature structure in the bone marrow** |
| **Stroma** | | **The area in between the microvasculature and the endosteal surface** |

**Table 3: Important terms used in this section and their definitions.**

### *Sources of cells*

Although so-called "mesenchymal cells" can be isolated from many different adult tissues, such cell sources remain to be vigorously verified (the need for verification

is discussed later in this sub-chapter). In this section some concepts of cell sourcing of interest to the field will be discussed.

It is highly unlikely that for human clinical application, non-human cells would be used. However, once human cells have been settled upon, the question of autologous (self) vs. allogeneic (non-self) cell source becomes critical. In Chapter 3, successful transplantation of allogeneic cells is observed in FVB-N/J mice. These mice are highly inbred, and therefore do not have an immune response to the transplanted cells, despite being immune-competent. However, humans are not inbred and therefore are much more likely to display a tissue rejection response. Some researchers claim that bone marrow stromal cells are immune-privileged, i.e., will not be rejected in an allogeneic setting [179], and while this may be true when injected into an embryo before the development of a competent immune system, other researchers have shown that when injected systemically, allogeneic BMSCs do, in fact, get rejected [180]. They are even more likely to do so as the cells differentiate and express histocompatibility antigens. Thus, autologous cells should optimally be used, in order to avoid necessitating immune-suppression [181].

If we assume, for the moment, that the best source of autologous regenerative cells is from bone marrow, then studies have shown that the location of donor bone and host bone can be very important. Studies have shown that bones in the facial region should be reconstituted from bone marrow derived in this region, and bones in the axial skeleton (including the cranial vault) and appendicular skeleton, from bone marrow derived from those. These two skeletal regions have different embryonic origins. The former derive from neuroectoderm, whereas the latter are derived from mesoderm [181]. Studies have demonstrated a certain level of incompatibility when cells from the iliac crest are applied towards jawbone regeneration [182], although the exact mechanisms have yet to be elucidated.

Two promising sources of cells, which will be briefly discussed here, but are not utilized in this project, are human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs). Both, in theory, have a much higher ability to proliferate and deliver much higher numbers of differentiated cells, with a much higher capacity for producing varied cell types. Both of these however, are hindered by concerns.

hESCs are derived from the inner cell mass of the blastocyst and present the ability to produce large quantities of undifferentiated "true stem cells" (with the ability to both self-renew and proliferate in the undifferentiated state, as well as form cells of multiple lineages *in vivo*) [181]. Embryonic stem cells were first derived from primate embryos [183], and shortly thereafter from human embryos [184] in the 1990s.

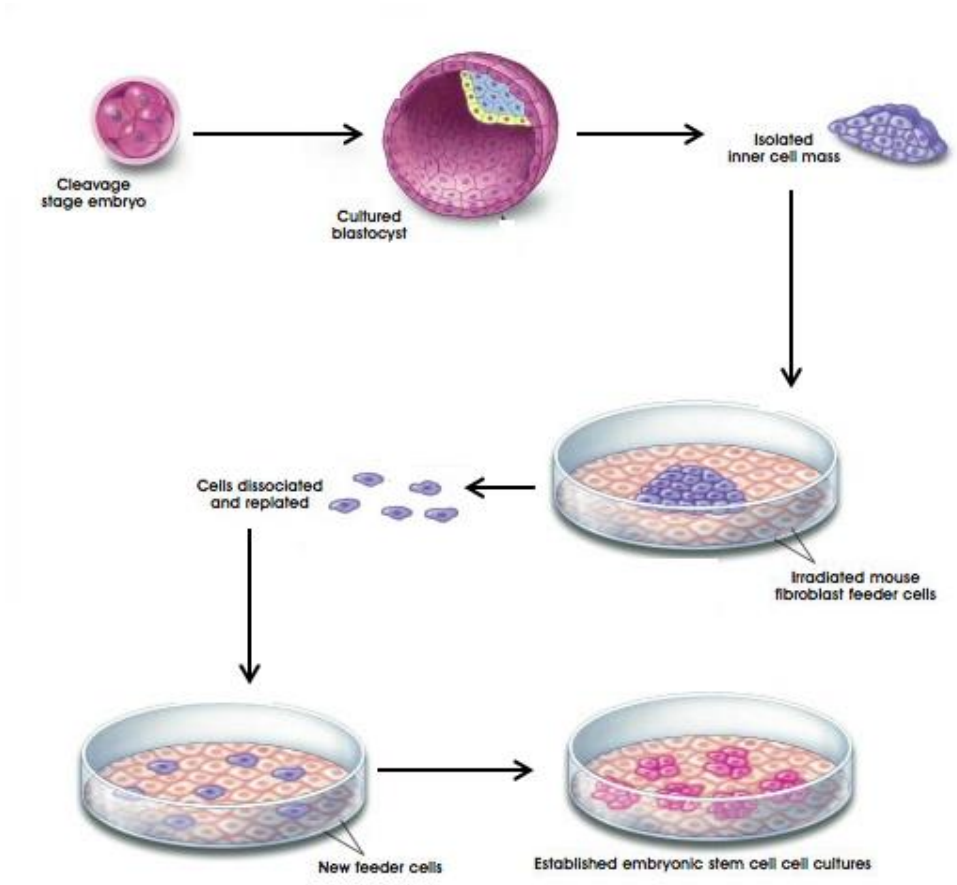



**Figure 10: The derivation of hESCs.** Modified from [185].

It is important to consider hESCs as addressing certain limitations presented by adult hBMSC treatment. hBMSCs must be transplanted at low passage, because it has been shown that long term *in vitro* culture effect gene expression in these cells; this means that cell numbers are limited due to the number of stem cells (multipotent CFU-F) harvested from a patient sample (for autologous transplantation). However, hESCs can be proliferated to large numbers in an undifferentiated state – and then only treated to differentiate towards the osteogenic lineage when desired. While many studies have focused on the ability of such cells to osteogenically differentiate *in vitro* (measured via the osteogenic assay discussed, and performed with hBMSCs later in this thesis), the *in vivo* results from the available cell lines have been less than promising to date [186, 187]. hESCs are hindered by the requirement for histocompatibility matching, since any transplanted material would be allogeneic. Additionally, the ethics of sourcing, and strict regulations surrounding research using hESCs (though these vary by country) restrict the ability of scientists to study the true viability of hESC treatment as a clinical option.

Induced pluripotent stem cells are considered to be similar to the autologous equivalent of ESC. Cells would be taken from the patient and treated such that the cells return to a stem-like state, known as induced pluripotency This process was first applied to murine [188] and then human [189] cells by the group of Shinya Yamanaka (Nobel Prize in Medicine 2012 for this work).

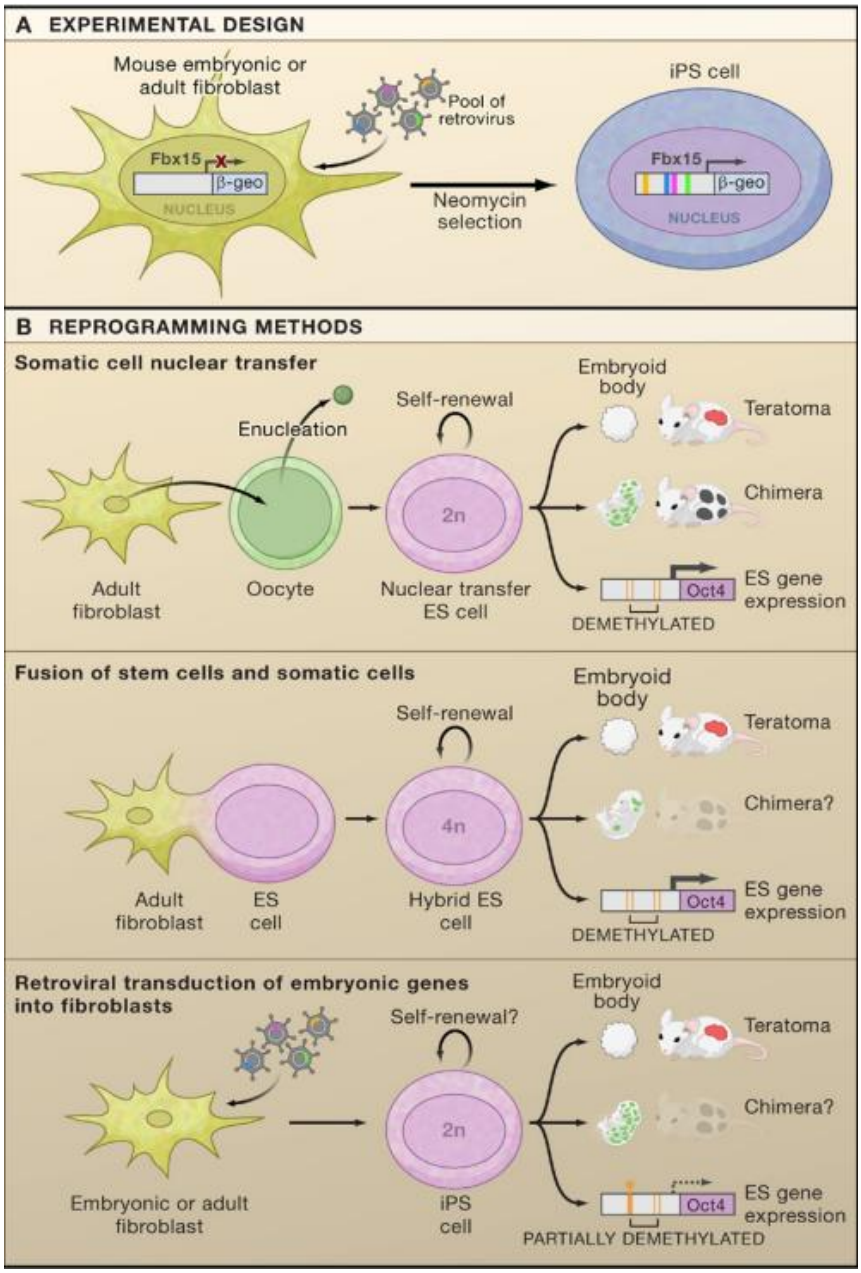



**Figure 11: The general concepts for the reprogramming of mature cells into iPSC and then driving differentiation.** A) Embryonic and adult mouse fibroblasts expressing a selectable marker (β-geo_ driven by an ES cell-specific promoter (*Fbx15*) are transduced with retroviruses encoding different candidate reprogramming factors. After selection with the antibiotic neomycin, drug-resistant β-galactosidase-positive cells are identified. These cells, called iPS, have many of the characteristics of embryonic stem cells. B) Methods of nuclear reprogramming and their outcomes. (Top) In somatic cell nuclear transfer, a somatic cell nucleus is introduced into an enucleated oocyte and used to produce mouse ES cells. These nuclear transfer ES cells are completely reprogrammed: they self-renew, are pluripotent (they form embryoid bodies and teratomas), can contribute to all germ layers of mouse chimeras, and express a full complement of ES-specific genes. (Middle) Fusion of ES cells and somatic cells is shown. A somatic cell is fused with an ES cell, leading to the reprogramming of the somatic cell's nucleus. Although these fused hybrid cells show self-renewal, are pluripotent, and express the normal complement of ES-specific genes, the ability to test their contribution to the chimeras remains difficult because of their tetraploid chromosome complement. (Bottom) Transduction of embryonic and adult mouse fibroblasts with retroviral vectors encoding embryonic transcription factors. Although the transduced fibroblasts are pluripotent, they only contribute to mouse chimeras up to E13.5 (no live chimeric pups have been obtained). Further, the transduced fibroblasts are not completely reprogrammed, exhibiting incomplete reactivation of ES-specific genes and only partial demethylation of the *Oct3/4* locus. Figure modified from [190].

The ability to revert adult differentiated cells gives iPSCs an advantage over hESCs because of the ability to use autologous cells. Like with hESC, scientists aim to take these less differentiated cells, grow them to high numbers in an undifferentiated state, then treat them to drive them down the osteogenic pathway. It is postulated that many of the same differentiation pathways applied to drive hESC differentiation could also be used for iPSCs. iPSCs are hindered by concerns regarding reprogramming methods (including viral), the concern that the reprogramming is not complete (epigenetics and other genetic memory), and the stability of the resulting genome [181]. This field of research is very new, and is likely to continue to be the subject of much interest for many years to come.

### Stem Cells in Bone Development

It is imperative to discuss what is known about cells in bone development as a background to discussion of the cells that will be used for the tissue-engineering project presented below. The first concept of a skeletal stem cell, though not framed in those terms, comes from the groundbreaking paper by Tavassoli and Crosby in 1968 [191]. Ever since then, the quest to elucidate the location and nature of such a cell (or cells) has been continuously evolving – the subject of a vast body of research. Theories for this particular subject have changed significantly, even over the last five years.

The bone organ, as mentioned above in the Bone Development section, undergoes a relatively high rate of turnover as contrasted to other organs. This turnover and regeneration requires a host of different cell types – but where do these cell types come from? When considering bone organs as a whole, both the calcified bone tissue as well as the hematopoietic bone marrow tissue must be studied. Studies of heterotopic bone formation have found that it is not possible to form hematopoietic tissue without

osteogenic tissue, but that it is possible to form osteogenic tissue without hematopoietic tissue [192], although the osteoid without hematopoiesis is undesirable [181].

Tavassoli and Crosby showed that cells from bone marrow, without any other bone components, were able to form bone and marrow heterotopically [191]. Bone marrow consists of a plethora of different cell types, so the challenge ever since then has been to determine which subset of cells represents the stem cell that drives this process.

"Stem cells" must necessarily contain two abilities: the ability to undergo self-renewal (i.e., upon cell division either one or both of the cell produced remains in the original, less-differentiated state) under controlled conditions, but also the ability to differentiate into a variety of more determined, specialized, cell types and reform a tissue [7]. The logical next step from a whole bone marrow aspirate transplant is to study clonogenic cells to see if any of them represent a stem cell. If the whole bone marrow population mix of cells is plated at low density, then some of these cells will attach and begin to proliferate, forming colonies, each arising from a single cell. These colonies are said to come from colony-forming unit fibroblasts (CFU-Fs). Much of the original work on CFU-F determination was performed by Friedenstein *et al.* [193]. They were able to study populations derived from CFU-Fs from different tissues. Importantly, when transplanted using an *in vivo* renal capsule model, CFU-F-derived populations from bone marrow would form bone tissue, whereas CFU-F-derived populations from spleen formed reticular tissue [193]. CFU-F-derived populations can be characterized by gene expression, protein expression, and cell surface markers. Many researchers also perform *in vitro* assays to test for differentiation into a variety of cell types. But the real test of whether a colony derived from a CFU-F indeed contains a skeletal stem cell is whether or not the cell is capable of forming bone and supporting hematopoiesis when transplanted heterotopically *in vivo*. There are several take home messages from the existing literature [181]:

- Only some cells within the bone marrow population are adherent in cell culture
- Only a percentage of adherent cells are actually CFU-Fs and will proliferate to form a colony
- *In vitro* studies can be performed to push CFU-Fs to different cell fates and lineages; however many of these results are the result of artifacts and may not be reproduced *in vivo*
- Only a percentage of CFU-F-derived colonies will form bone *in vivo*
- Only a percentage of bone-forming CFU-F-derived colonies will both form bone and support hematopoiesis (10-20% of the total CFU-F number) [194]
- Performance of bone marrow aspirates varies from patient to patient in terms of both by raw numbers of CFU-Fs as well as by performance outcomes for a number of reasons

Now that a sub-population of cells was shown to be CFU-Fs, the question became one of finding markers that define this cell type. The first such marker found was CD146 (also known as melanoma cell adhesion molecule) [195]. CD146 has been shown to be highly expressed in all CFU-Fs isolated in culture. Therefore, the next step was to identify the natural location and identity of CD146 positive cells. Osteoblasts and other bone-lining cells *in vivo* do not express CD146. In fact, it has directly been shown that increased expression of osteoblastic markers (discussed in the next section of the Introduction) as cells differentiate is directly coupled with decreased expression of CD146. Rather, the cell population within natural bone tissue that expresses high levels of CD146 is actually known as adventitial reticular cells (ARCs).

ARCs are so called because they are adventitial, meaning that they are associated with the covering tissue of a blood vessel (specifically sinusoid) wall; and because they are reticular, meaning that their cell morphology is very branched, with tendril-like cell projections into the surrounding hematopoietic tissue [192]. Evidence for the role of

ARCs in hematopoiesis comes from both their morphological association with hematopoietic tissue as well as their expression of CXCL12, the ligand for CXCR4, a receptor that has been specifically associated with the hematopoietic stem cell microenvironment [196]. CD146 positive cells form bone and support blood formation when transplanted *in vivo*, as well as their marked expression of alkaline phosphatase [197]. Other markers linked to these ARC skeletal stem cells are, CD105, high alkaline phosphatase expression, and STRO-1 [198].

It has been shown that the hematopoietic tissue in heterotopic transplant experiments comes from the transplant host and not the seeded cells [196, 199, 200]. However, the formation of hematopoietic tissue depends on the presence of a vascular network organized by donor stromal cells [196].

In terms, therefore, of the successful co-development of bone and hematopoiesis, it can be seen that although ARCs are skeletal stem cells and not hematopoietic stem cells, they are nevertheless the driving force for recruiting actual hematopoietic stem cells to the area of bone regeneration. The role of these skeletal stem cells in order to organize and support hematopoiesis was first demonstrated by Friedenstein *et al.* [201], showing that the two stem cells were separate. Stem cell localization is throughout the bone marrow, particularly at areas of high micro-vascularization and sinusoid formation, which are areas that have long been observed to be linked to bone turnover. In the bone, therefore, we can think of cells at the endosteal surface (including osteoblasts and osteoclasts in areas of bone turnover, but also non-osteoblastic, perhaps post-osteoblastic bone-lining cells in other areas); the cells at the vasculature (i.e., the blood vessel walls supported by pericytes, some of which are ARCs); and of the cells in between (stromal cells), about which not much is truly known. That said, as the structure of bone and marrow can be quite intertwined, particularly in cancellous bone, the perivascular surface may also be directly adjacent to the endosteal surface, which

has led to confusion over the years [202]. This model does not apply to all bones in the skeleton; as hematopoiesis, while initially present throughout all bone types, gradually phases out from all bones until in the 20s of human life, when it localizes to specific areas in the axial skeleton and areas of the long bones. However, this hematopoietic ability is not irretrievably lost. Thus, when thinking about stem cells for both osteogenesis and angiogenesis, the concept of a stem cell cannot be limited to a cell that sits in a constant environment [203]. The flexible nature of this stem cell system at the adventitial surface is best described by this quote from Bianco *et al*. [203]:

***"A nonosteoblastic, and yet preosteoblastic, and nonendothelial, and yet periendothelial cell resides at the abluminal surface of sinusoids in the human BM in vivo."***

In relation to cells in the perivascular region and cells in the endosteal region (discussed in the next sub-section), we should perhaps briefly discuss the stromal cells mentioned earlier. Very little is known about these cells, even less than about cells on either of the other two surfaces. But again, as mentioned, the entire stromal structure is incredibly complex and although these cells are not perivascular, they are certainly closely associated with the pervasive anatomy of bone marrow, and therefore likely to play supporting roles in both osteogenesis and/or hematopoiesis as well as adipogenesis [192]. Figure 12 below succinctly illustrates the cell biology concepts explained above and the possible cell culture options [198].

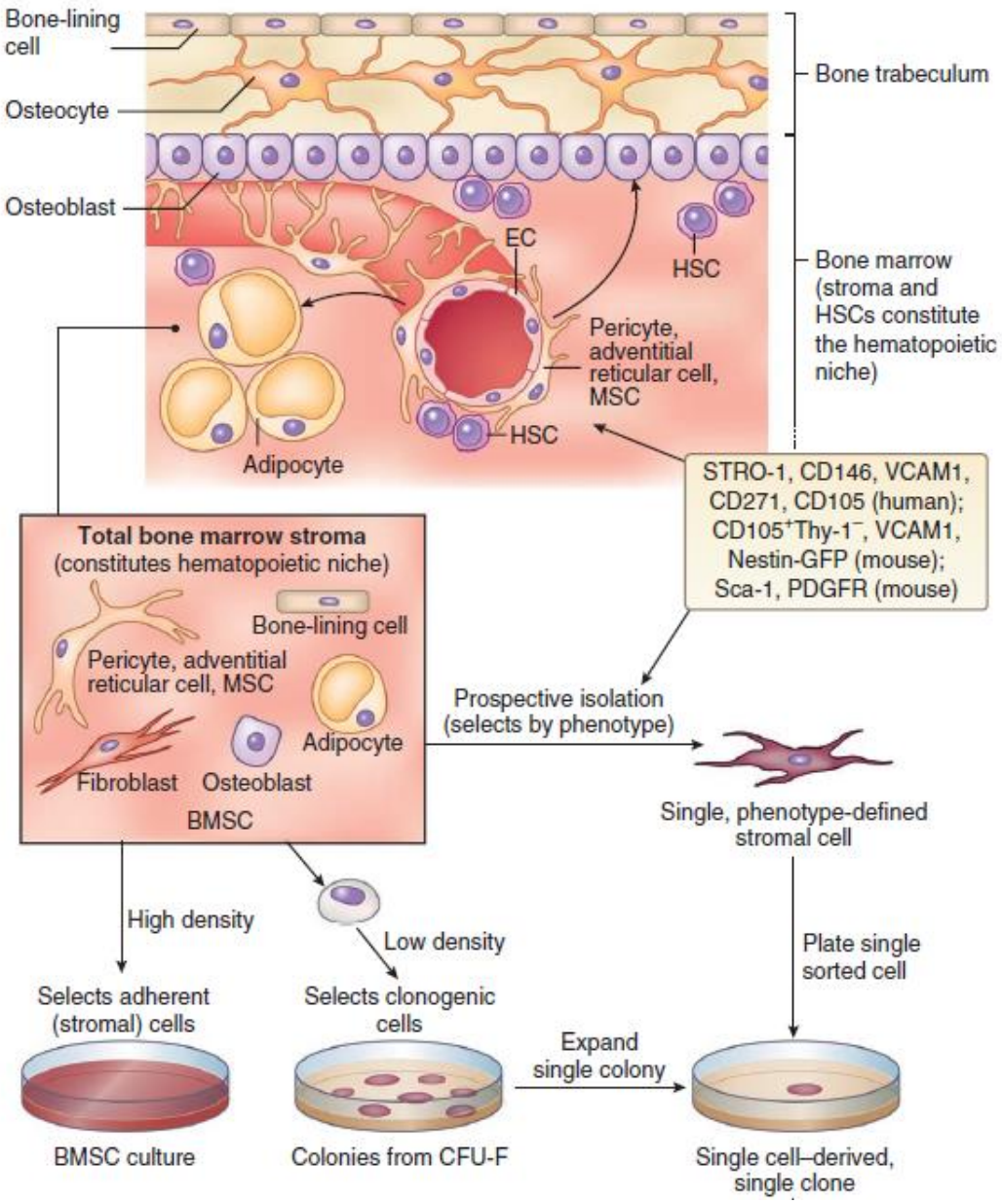



**Figure 12: The whereabouts of MSCs.** In the postnatal bone marrow, ARCs reside around sinusoids, maintain a niche for HSCs, support hematopoiesis and replenish the differentiated compartment of osteoblasts and adipocytes during tissue growth and turnover; they also generate cartilage under specific conditions such as trauma. Total populations of bone marrow stromal cells (BMSCs), which do not coincide with cultures of stem cells in any way, are established when total bone marrow cell suspensions are plated in culture at high density. Adherence to plastic separates the stromal cells from non-adherent hematopoietic cells. If cell suspensions are plated at low density, only cells capable of density-insensitive growth are selected (CFU-Fs). These cells initiate the growth of clonal colonies. Passaging a single colony or, alternatively, establishing a clonal culture from single cells isolated on the basis of their expression of phenotypic markers, which may be different in human and mouse, results in a clonal population. Modified from [198].

## A Brief Tangent: Mesenchymal Stem Cells vs. Skeletal Stem Cells

The term "mesenchymal stem cell" was first introduced by Caplan in 1991 [204]. If we take the term at face value, then a mesenchymal stem cell should be derived from mesoderm and should be able to differentiate into the whole range of mesoderm-derived cell types. Research in mesenchymal stem cells has exploded, as evidenced by a PubMed search for the term.

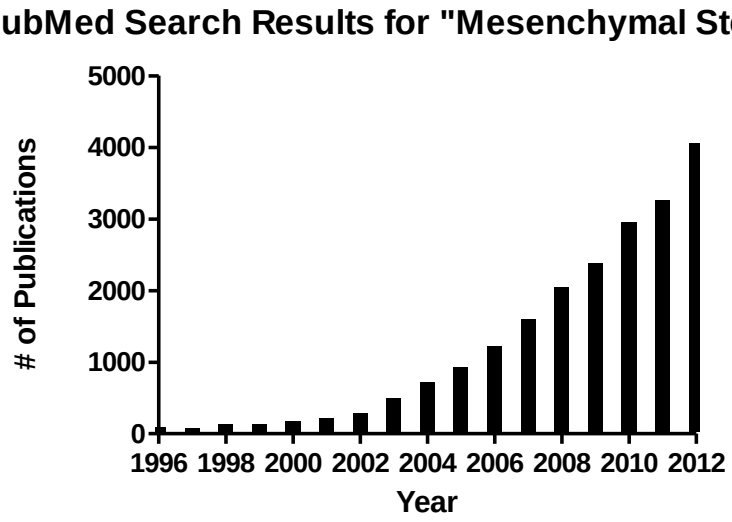



**Figure 13: The "explosion" of published articles which feature the term "mesenchymal stem cell".** Figure created from [205].

Unfortunately, many of these publications almost certainly incorrectly use the term "mesenchymal stem cell" (MSC). From the viewpoint of this project it is important to look at literature pertaining to two different topics. The first of these is related to cells derived from sources other than bone marrow that researchers claim to be osteogenic. The second is related to claims that cells derived from bone marrow are capable of differentiating into cells outside of the skeletal cell family. Bone marrow cell differentiation into non-skeletal tissues is not discussed here, as the present thesis is focused on the formation of bone. Figure 14 below demonstrates the range of definitions, usages, and implications of the term MSC from the strict (Yin) to the most relaxed (Yang) interpretations [198].

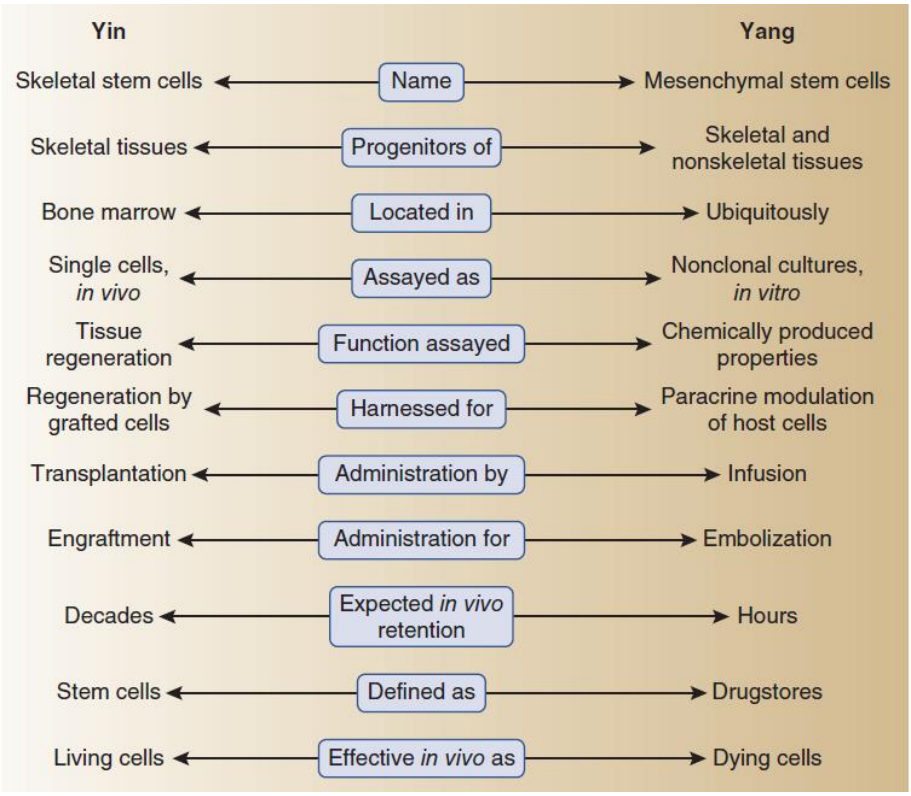



**Figure 14: The "Yin and Yang" of the "mesenchymal stem cell".** Figure adapted from [198].

Although the ability of non-bone-marrow-derived cells to differentiate into a bone organ has been claimed, many of these assays rely on *in vitro* assays, which can often be pushed by the use of high levels of β-glycerol phosphate into a state of dystrophic calcification. While this might result in a highly calcified result in the petri dish, the need for *in vivo* confirmation is paramount. Many of the tissue sources that have been tested *in vivo* have been tested with the additional application of bone morphogenetic proteins (BMPs). This assay, while often capable of forming calcified tissue, is nevertheless not a representative model. BMP on a transplanted scaffold without any seeded cells has been shown to form osteoid by itself through induction of local cells, so the addition of cells to this system does not prove that the cells themselves cause osteogenesis. Finally, the major problem with these *in vivo* assays performed under the influence of BMP is the often-observed lack of hematopoiesis in such transplants. The lack of hematopoiesis is not trifling, as hematopoiesis formation is

indicative of the presence of the dual stem cell perivascular space discussed above. Without this "niche" bone regeneration will be a one-time event, but the bone formed will not be capable of bone regeneration during turnover, as all cells irreversibly osteogenically differentiate and none remain in stem cell form [181]. This ability of certain cell types to form osteoid or osteoid-like structures without supporting hematopoiesis has been demonstrated for cells derived from adipose [206], dental pulp [182], and periodontal ligament [207].

On the other hand, cells derived from the bone marrow stroma have been definitively shown to possess the ability to both develop osteoid and support hematopoiesis, as well as to self-renew [181]. So how is the true skeletal stem cell defined? As mentioned previously, much work has been done to identify markers of this skeletal progenitor, with Stro-1 and CD146 being the primary markers. Some of the markers from the current literature either demonstrated to be present on, or absent from, the putative skeletal stem cell are shown below [7]:

| Cell-surface antigen | Expressed? | Details |
|---|---|---|
| **Stro-1 antigen** | **YES** | **Cell-surface trypsin-resistant antigen expressed by CFU-F, Antibody: STRO-1** |
| **Tissue non-specific alkaline phosphatase (TNSALP)** | **YES** | **Cell-surface glycoprotein associated with osteoblast lineage cells, Antibody: STRO-3** |
| **CD63** | **YES** | **Synonyms: Melanoma-associated antigen ME491, Tetraspanin 30, Lysosomal-associated membrane protein 3, Antibody: HOP26** |
| **CD105** | **YES** | **Synonym: endoglin/SH2** |
| **CD73** | **YES** | **Synonym: SH3/4** |
| **CD71** | **YES** | **Synonym: Transferrin receptor protein 1(TfR1)** |
| **CD90** | **YES** | **Synonym: Themocyte antigen-1 (Thy-1)** |
| **CD49a** | **YES** | **Synonym: Laminin and collagen receptor VLA-α1** |
| **CD106** | **YES** | **Synonym: Vascular cell-adhesion molecule-1** |

| Cell-surface antigen | Expressed? | Details |
|---|---|---|
| | | **(VCAM-1)** |
| **CD166** | **YES** | **Synonym: Activated leukocyte cell-adhesion molecule (ALCAM), Antibody; SB-10** |
| **ICAM-1** | **YES** | **Intercellular cell-adhesion molecule-1** |
| **CD29** | **YES** | **Synonym: β1 subunit of the integrin family** |
| **CD31** | **NO** | **Synonym: Platelet/endothelial cell-adhesion molecule-1 (PECAM-1)** |
| **CD56** | **NO** | **Synonym: Neural cell-adhesion molecule (NCAM-1)** |
| **CD18** | **NO** | **Synonyms: Leukocyte cell-adhesion molecule, integrin β2 (LCAMB), Lymphocyte function associated antigen-1 (LAD, LFA-1)** |
| **CD45** | **NO** | **Synonym: Leukocyte common antigen. Located on hematopoietic cells except erythrocytes and platelets** |
| **CD34** | **NO** | **Identified on hematopoietic progenitors and endothelial progenitors** |
| **CD14** | **NO** | **Preferentially expressed on monocytes/macrophages** |
| **CD11** | **NO** | **α component of various integrins, especially those in which the β component is CD18** |
| **CD44** | **NO** | **Expressed by hematopoietic as well as bone marrow mesenchymal stem cells** |

**Table 4: List of important markers proven to be present or not present on skeletal progenitor cells.** Table adapted from [7].

In addition to the markers listed above, which were mostly identified through FACS and immunohistochemistry, other methods, including hybridoma technology and phage display methods, have been studied to improve skeletal stem cell isolation, [208]. However, the aim of this thesis is not to specifically isolate skeletal stem cells, but rather to use the bone marrow cell population as a whole; hence, stem cell isolation methods will not be discussed further here.

## Osteogenic differentiation

As with research into the stem cell "niche" discussed above, there is a huge, complex, and ever evolving body of knowledge about how bone-related lineages derive from their progenitor cells. This section primarily provides an overview of the differentiation of osteoblasts and osteocytes from skeletal stem cells. As mentioned previously, bone is an organ that undergoes a relatively high rate of turnover. At any given time, 0.1-7.3% of the endosteal surfaces of bone are undergoing bone regeneration [104]. As hinted at previously, although endosteal surfaces are constantly covered with bone-lining cells, the presence of osteoblasts actively laying down bone (the definition of an osteoblast) is limited to areas of regeneration. Regenerative pockets are present for only roughly 3 weeks, after which the cells then either migrate from the area, become osteocytes, or become quiescent to form bone-lining cells [203]. This small area of remodeling has proven difficult to study as, while it is easy to morphologically tell the difference between osteoblasts and other bone-lining cells *in vivo* (the former are actively laying down bone, whereas the latter are flat, elongated and relatively inert), there has been confusion in the literature between osteoblasts and cells of osteogenic lineage, and the markers with which to define different stages of maturity [192]. While all osteoblasts are of osteogenic lineage, there are also several other cell types that are also derived from the skeletal stem cell (e.g., cells involved in bone, stroma, marrow, adipocytes, and cartilage). Unfortunately, this pathway is not as linear as many other cellular differentiation pathways in human biology because the modulation of cells between different phenotypes is often not irreversible. Also, cells can share large portions of differentiation pathways and be localized to the same areas of tissue, and yet perform very different biochemical functions [203].

The following figure shows the results of genes found to be associated with osteogenic phenotypes (particularly bone mineral density) through genome wide association studies [209].

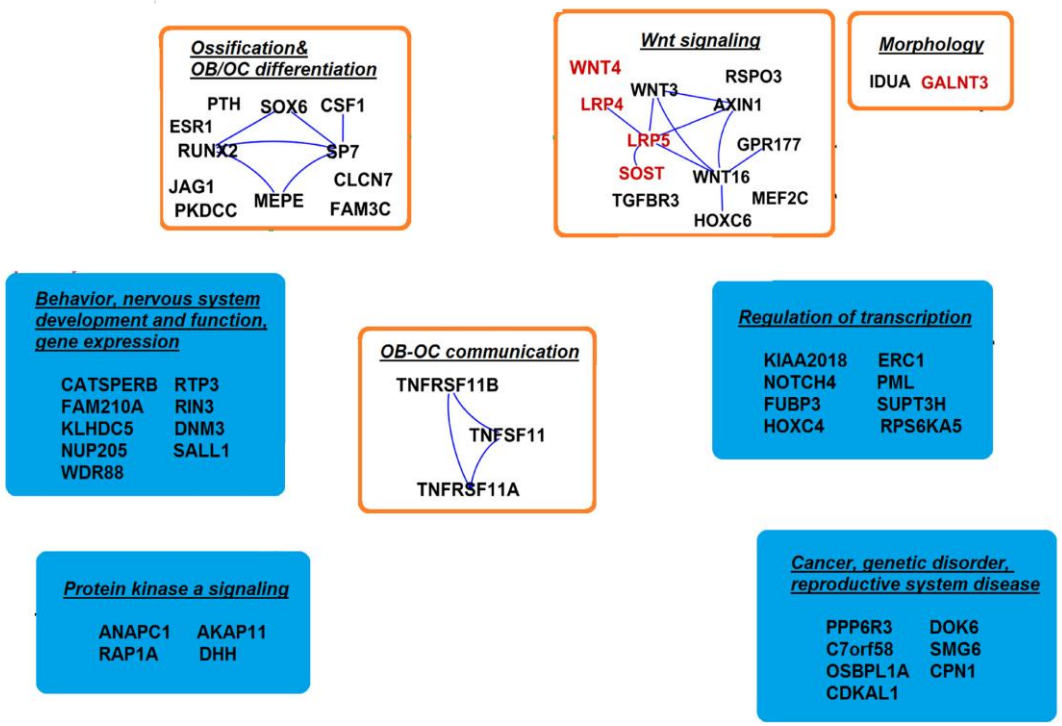



**Figure 15: Genes linked in GWAS studies to bone mineral density, grouped by their biological pathways and functions. Boxes with orange borders are skeletal pathways or skeletal gene groups, whereas blue boxes are other pathways or gene groups not specifically skeletal. OB = osteoblast, OC = osteocyte.** Modified from [209].

Major gene expression pathways found to be correlated with different stages of bone differentiation are shown below [210]. These are markers commonly used by the researchers in the field, and are included in qPCR studies presented in Chapter 5.

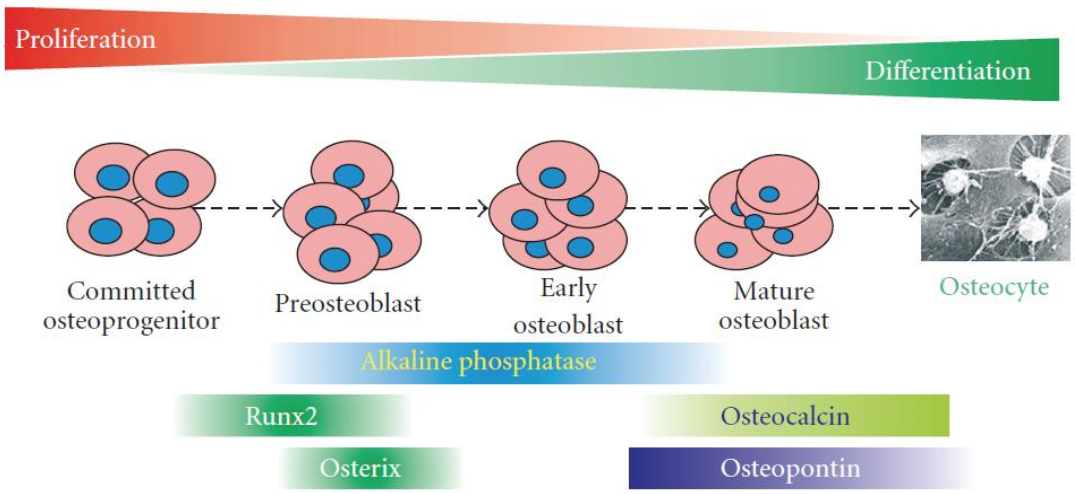



**Figure 16: Important markers of osteogenic differentiation. Note the high level of expression overlap.** Modified from [210].

## Bone Marrow Stromal Cells for Our Experiments

The term bone marrow stromal cells (BMSCs) used throughout this thesis is a specific term related to a group of cells in culture. Specifically, "BMSCs" refers to the rapidly adherent cell population cultured from whole bone marrow. Thus, while the term "BMSCs" does include the cells described above as the cells between the perivascular and endosteal spaces, it also includes any cells from these other two cell spaces, that were part of the marrow flush and were adherent. This definition explains why BMSCs are considered a mixed population of cells and why CFU-Fs (the *in vitro* equivalent to ARCs) are a subset of cells within the BMSC population.

While BMSCs can be isolated from iliac crest aspirates, core biopsies and surgical waste, the former are often contaminated with high levels of peripheral blood, which can negatively impact culture. Bone segments from biopsies and surgical waste, when handled properly by all parties (from surgeon to scientist) can be used to derive osteogenic cells through enzymatic and mechanical methods, as well as through explant cultures [181]. The main drawback for using primary-derived BMSCs is the limited number of cells obtained from harvest combined with the desire to keep cell passage low. Cells can be expanded *in vitro* for either patient treatment or scientific research, but studies have shown that BMSC gene expression changes over successive passages, and therefore minimal processing is desired.

Studies have shown that hBMSCs are vital for the induction of neovasculogenesis *in vivo* [211-213] in a method dependent on their microenvironment; e.g., growth factors, ECM, and mechanical properties of their surroundings [214]. hBMSCs have been found to be particularly responsive to VEGF [215], PDGF [214], and hypoxia [216]. In addition to these properties of hBMSCs and their ability to give rise to a variety of skeletal cells, hBMSC populations include some cells which have the ability to self-renew (skeletal stem cells), allowing for continued cell function and turnover with time, not

just the immediate capability to heal a bone defect [41]. Studies which apply hBMSCs to long-term bone-related procedures have met with considerable success [217]. Additionally, the application of hBMSCs to non-bone related disease states; e.g., ischemic heart patients, has demonstrated the vast healing potential of these cells through paracrine effects [218].

### Endothelial Cells

Endothelial cells (ECs) are crucial for forming the lumen of blood vessels and have been successfully applied during various treatments of vascularization disease states [219]. ECs were originally thought to serve a strictly structural purpose in lining blood vessels, but now it is understood that they regulate vascular processes in a number of ways [220]. For example, human umbilical vein endothelial cells (HUVECs) on collagen-gel constructs have been shown to demonstrate non-thrombogenic properties including delayed clotting time and reduced platelet microparticle formation [221]. The presence of ECs (indicating assumed angiogenesis) has been shown to be crucial for the recruitment and survival of supporting tissue cells [222]. Although studies convincingly indicate that it is possible to extract bone marrow-derived ECs and endothelial progenitor cells from adult patients [223], the availability of these cell types is low compared to the ease with which HUVECs can be obtained.

### Co-Culture of Cells

Many different *in vivo* and *in vitro* studies have been conducted on the use of two or more cell types together. The aim of using multiple cell types is to mimic the complex interactions between cells that would not be present in single cultures [49]. For the purposes of the experiments described below, co-culture of hBMSCs and ECs was employed. The optimum combination of cell types and their ratios (as well as any other factors, such as culture conditions) for use in 3D scaffolds has yet to be determined [49, 224, 225]. General belief in the field of tissue engineering, is that the co-culture of cell types within a scaffold will allow for the formation of vasculature within the scaffold,

though studies differ about whether direct co-culture or merely conditioned media is required. A blood vessel structure cannot survive without surrounding supporting perivascular and stromal tissue that secretes supporting matrix and stimulating growth factors. This need for supporting structure can, in part, explain why VEGF is more vital for vessel survival in the embryo, whereas adult blood vessels are less dependent on VEGF supply and can survive in a quiescent state for a number of years [56]. Studies include the co-culture of ECs and fibroblasts [226] and even the treatment of ECs with medium that was fibroblast-conditioned resulting in EC elongation, and development of capillary-like structures (CLS) [227]. Additionally, a combination of keratinocytes, dermal fibroblasts and HUVECs was shown to spontaneously form CLS given the right microenvironment [228]. Studies which combined HUVECs and hBMSCs were shown to recruit hematopoietic and osteogenic progenitors to the site of bone remodeling and effect their differentiation [5].

## Growth Factors

### Overview

Growth factors are signaling molecules which regulate cellular growth and function [229]. These factors can either up-regulate or down-regulate various cellular processes in a dose-dependent fashion through interaction with cell-surface receptors [230]. In this overview section, the focus is on growth factors in the context of bone formation and fracture healing. Some factors discussed below and shown to be related to the natural healing of fractures include bone morphogenetic proteins (BMPs), TGF-β, IGF, FGF, PDGF and VEGF [10, 68]. Multiple factors are involved in bone induction in a dosage-dependent specific temporal and spatial arrangement and act synergistically [1, 2, 109, 230]. It follows naturally that scientists would look at application of these growth factors in order to improve performance of tissue engineering therapies [56, 156].

Traditionally, angiogenic and anti-angiogenic factors have been identified and studied using various models, including, but not limited to, the chorio-allantoic membrane assay and various corneal and iris vascularization assays [157, 231-236]

The evaluation of growth factors for their possible use in tissue engineering follows a common pattern of research [71]:

1) the effect on cell cultures

2) the effect in small animal experiments

3) the effect in large animal experiments

4) observations on controlled series of patients

5) randomized controlled trials in patients

The delivery of growth factors to a system is often achieved through a simple injection of a single growth factor in solution; however, this method results in non-targeted distribution of the growth factor, and although successful in some instances in animal models, such injections have not shown much success in the clinic [5]. The research goals in this thesis, as discussed above, are centered on a cell and 3D scaffold approach. Scientists have documented the significant augmentation of the cell and scaffold approach by the addition of growth factors, not only through improved osteogenic histology, but also increased angiogenic and vasculogenic processes necessary for complete success of the implant [1, 38]. Extracellular signaling molecules can be categorized as either soluble or immobilized to the ECM (through predominantly ionic interactions) [237].

The effective concentration of a growth factor at any given point in time is dependent on the rate it is being synthesized and secreted from local cells, the rate at which it is coming into the system, the rate it is being broken down by various proteases,

the rate at which it is being cleared from the system, and the extent to which it is being inhibited by antagonistic molecules or simply in inactive form due to lack of agonistic molecules, as shown in Figure 17 [38, 220].

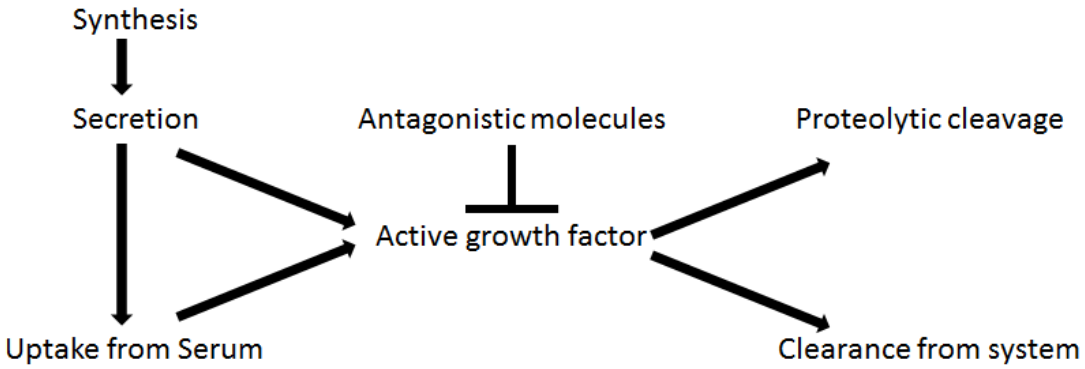



**Figure 17: Dynamics of growth factors.**

Another reason for the delivery of growth factors on a 3D scaffold is that the scaffold not only concentrates the growth factor in a small specific area where osteogenesis is desired, but also prevents the spread of growth factor to areas where osteogenesis is not desired [230]. The diffusion of growth factor to areas where osteogenesis is not desired could lead to adverse side effects as many of the project growth factors are involved in processes outside of osteogenesis [38]. Indeed, by acting to stimulate the formation and stabilization of an immature vascular structure and to inhibit apoptosis, many of the growth factors are known to be over-expressed in tumorigenesis. This relationship of over-expression to tumorigenesis is one reason why gene therapy to induce cells to directly over-express certain growth factors is controversial [38]. However, such methods are not at all uncommon [2, 238-243]. In addition to the delivery of growth factor localized to a 3D scaffold, the delivery of growth factors in carrier capsules that release them over a timeframe have been much studied [2, 71, 230]. Although the use of applying several different growth factors to a system simultaneously has often been shown to act synergistically [244-246], factor identity

and dosage should be chosen carefully in order to avoid inhibition of osteogenesis [247]. In addition to using growth factors, various other molecules; e.g., self-assembling peptides, have been tested either as coatings for scaffolds [141], or in injectable form to encourage an osteogenic microenvironment. An additional complication in tissue engineering arises from the interaction of cells with the scaffold material, as multiple studies have shown that transcript levels of growth factors by cells changes upon contact with biomaterials as contrasted to 2D culture on tissue culture plastic [248-253].

### Transforming Growth Factor β-1

The TGF-β family of proteins is ubiquitously expressed but is predominantly localized to bone and platelets [254]. The protein is originally secreted in a much larger precursor form and proteolytically cleaved to form active TGF-β dimers. The different compositions of the TGF-β isomers in the dimer determines binding affinities and hence bioactivity. Different isoforms are found to exhibit regionality within the body and specific tissues [10]. TGF-β1 is more often found in areas of osteogenesis whereas TGF-β2 and TGF-β3 are more often found in areas of chondrogenesis [255]. TGF-β in the extracellular matrix is sequestered by proteoglycans and glycoproteins that protect them from rapid degradation. TGF-β that is not ECM bound becomes associated with α2-macroglobulin and is cleared from the system [256]. The primary role of TGF-β is thought to be to expand the population of osteogenic precursors by binding to several different receptor types. Receptor types I and II are serine-threonine kinases which couple to various signaling pathways. The type III receptor is a non-signaling proteoglycan thought to provide a method for TGF-β sequestration, and the type IV receptors, also serine-threonine kinases, are specific to certain TGF-β superfamily subtypes, especially BMPs [109]. TGF-β also plays a key role in Smad-mediated signaling (discussed further in the BMP section below). TGF-β expression is regulated through feedback loops that affect only the specific signaling factor (e.g., TGF-β1 stimulates more TGF-β1 expression) and also by various types of hormone signaling.

TGF-β1 expression is increased during bone repair in a spatial and temporal-specific fashion. TGF-β1 is first expressed by inflammatory cells at the site of injury, followed by expression in osteoblasts, primitive progenitor cells, and sequestered in connective tissue near the site. At this stage, TGF-β1 serves the dual purpose of osteoprogenitor cell recruitment and proliferation, while also inhibiting the proliferation and differentiation of osteoclastic cells (to allow bone formation) [38]. As healing progresses, TGF-β1 becomes expressed solely by osteoblasts surrounding remodeling bone [109]. Here, TGF-β1 is vital for the remodeling of the area, while the breakdown of pre-existing bone releases additional TGF-βs into the system to stimulate osteogenesis [257]. TGF-βs also promote production of collagen and other ECM proteins, which provide a scaffold for ossification as well as directly inducing alkaline phosphatase expression (promoting ossification by removing pyrophosphate) [258]. TGF-β accumulation in bone has been found to diminish with age [259], which may affect regenerative capacity. Augmented ECM accumulation is TGF-β-stimulated through the increased synthesis of matrix-metalloproteinase inhibitors [109]. TGF-β application to an endothelial progenitor cell seeded synthetic scaffold placed in a bioreactor resulted in more organized osteogenic histology compared to controls [260].

TGF-β1 is the most abundant isoform of TGF-β in mammals, and also the most studied isoform [261]. Moreover, it is also the most abundant growth factor found in human bone [262]. However, results from bone-related experiments involving TGF-β1 are inconclusive. Clarke *et al*. [156] used a TGF-β1-loaded Gelfoam™ coating on stainless steel for a defect study. They found more active marrow in groups with low doses of TGF-β1 versus controls. While they thought this did not translate to more bone in the defect, the highest dose of TGF-β1 formed more vascularization and clot formation, but less tissue formation. Others have reported increases in positive bone qualities associated with the use of TGF-β1 [263-265]; yet others have reported negligible and sometimes even detrimental effects of TGF-β1 [266, 267], while still

others have postulated that TGF-β1 might only have a significant difference at early stages in bone repair, which might not be observable because studies analyze later time points [268]. In addition to temporal efficacy, TGF-β1 also appears to be more potent at lower concentrations, e.g., 0.1-20ng/mL *in vitro*, with efficacy decreasing and potential negative effects increasing once that threshold level is past [269, 270]. High levels of TGF-β1 expression are also associated with numerous health conditions both in humans such as glomerulus-related injuries and chronic multiple-sclerosis [271, 272] and in animals [273]. Another concern is whether growth factor should be administered as a large bolus at the start of the experiment or at a low concentration throughout [262, 269], although this concern loses some relevancy for *in vivo* experiments due to the logistics involved in multiple growth factor administrations. Multiple approaches have been taken for growth factor administration, including release from microspheres [274] and genetic engineering [275]. As for growth factor release kinetics from scaffolds, many different studies have been performed on various materials [266, 276-278].

### Vascular Endothelial Growth Factor

The VEGF family of glycoproteins consists of six main members, each of which has several splice forms [1, 279]. Different VEGF isoforms may contain exons that are heparin binding. VEGF signaling is considered a rate-limiting and necessary step in the initiation of angiogenesis [280]. The role of VEGF in angiogenesis, as hinted at above, is as a destabilizing force. By undermining the vessel basement membrane through the production of proteases and plasminogen activators, VEGF allows for endothelial cell proliferation and migration [281, 282]. Increased vascular permeability [279] also results in vasodilation which allows for increased delivery of growth factors and circulating cells [109]. Since angiogenesis, as described above, is crucial for bone regeneration, VEGF can be said to be the most predominant growth factor in both physiological and pathological angiogenesis [1, 90, 230, 279, 283-285]. Embryos that have abnormally high (or exogenously added) VEGF or impaired VEGF expression can

show dramatic abnormalities with regards to vasculature formation [286]. Even mice heterozygous for VEGF deficiency die at embryonic day E11-12 and mainly show problems with early vasculature formation [287, 288]. In most developing tissues the forming vasculature has been found to stain positive for VEGF receptors [289], whereas the surrounding stromal cells express VEGF [290, 291]. In addition to the stimulation of angiogenesis through interaction with endothelial cells, VEGF is involved in the recruitment of osteoblasts and osteoclasts through endothelial cell signaling cascades [292], as well as through direct endothelial cell-osteoblast cell-cell contact [293]. VEGF binds to two different receptors (VEGFR1 and VEGFR2), both of which are tyrosine kinase receptors. VEGFR1 has a higher affinity for VEGF, but a lower biochemical activity, which ensures that VEGFR2 is only activated under higher concentrations of the ligand [109, 279]. VEGFR1 is mostly correlated with cell differentiation and vascular organization, whereas VEGFR2 is more associated with EC proliferation [294]. VEGFR2 deficient mice do not form ECs or vascular structures at all and die at embryonic day E8.5-9.5 [56, 295]. VEGFR1 mice also die at E8.5-9.5, but have a vasculature, albeit a grossly abnormal one [296]. Although the VEGF regulatory system is not very well characterized, many growth factors discussed here are known to have effects on the VEGF pathway and vice versa; e.g., BMPs, FGF, PDGF and Ang-1 [214].

Recent studies show that VEGF may bind and signal through PDGF receptors, which are highly homologous to VEGF receptors (i.e., both are class III tyrosine kinase receptors). Evidence for this relationship comprises two parts. First, hBMSCs derived from healthy donors did not express VEGF receptors. Secondly, inhibition of PDGFR using specific antibodies or siRNA knockdown resulted in VEGF-induced BMSC migration and proliferation [214].

Various cell types produce VEGF, including macrophages, smooth muscle cells, BMSCs, and certain tumor cells [214]. The concentration of VEGF in the blood is related

to the vascular permeability of healthy tissue, but is insensitive to vascular permeability in pathological tumor tissue or to secretion of VEGF by tumors [258]. VEGF production is primarily induced through the formation of a hypoxic environment either *in vivo* or *in vitro* [56] which leads to the up-regulation of hypoxia-induced transcription factor (HIF). A hypoxic environment forms following fracture, which causes the release of lysosomal enzymes from necrotic bone and soft tissues as well as vasoconstriction, which results in a localized hypoxic area that stimulates neovascularization [109]. In addition to the stimulation of vascularization, VEGF has been shown to be an inhibitor of apoptosis [279] through several different pathways, including: Bcl-2 over-expression, PI3-K/Akt signal transduction, inhibition of SAPK/JNK24, and activation of MAPK/Erk [68]. As well as inhibiting osteoblast apoptosis, VEGF has also been shown to increase alkaline phosphatase expression in osteoblasts [297]. As is the case for Ang-1 (angiopoietin-1), VEGF (as well as its receptors) have become a target for anti-angiogenic therapies for tumors [298, 299].

As with many other tissues, the formation and repair of bone is highly dependent on VEGF expression and regulation [78, 300-304]. For example, scientists have found that both osteoblast VEGF production and endothelial BMP-2 production are regulated by the local VEGF concentration, which suggests a pathway that may reinforce osteogenic commitment and enhance bone repair [60, 305, 306]. VEGF, therefore, serves as a crucial signaling molecule between osteoblasts and ECs [307-311], and so it comes naturally that VEGF is a widely studied cytokine for use in tissue-engineered constructs.

The same concerns and methods apply with regards to VEGF application to a tissue-engineered scaffold as discussed in the TGF-β section with regards to delivery methods, dosages, and interaction kinetics. 2D cultures of HUVECs demonstrated that the addition of VEGF to the base coating of the surface greatly increased HUVEC

proliferation [312, 313]. Simple bolus injections of VEGF were shown to be eliminated from the physiological system within the space of 1 hour [314, 315], hence a more complex application method is necessary. A mouse model in which VEGF was inhibited near a fracture resulted in impaired bone formation [316]. Conversely, the application of exogenous VEGF to a fracture in a rabbit was shown to enhance bone repair [79]; this result is not surprising, since a similar application of exogenous VEGF was shown to increase blood flow to the fracture zone five-fold [317]. Experiments with conditional knockouts of VEGF have led to lack of bone formation during embryogenesis and impaired bone regeneration in adult animals [109]. At the other end of the scale, over-expression of VEGF has been linked to the growth of aberrant blood vessels and hemangiomas [318]. Thus, a carefully chosen concentration of VEGF is needed to produce a more normal capillary structure [71]. Investigators have conducted studies on the effect of introducing VEGF to allografts, since processed allografts often do not become well vascularized [8]. Studies in which a combination of PDGF, TGF-β, and FGF, were applied to a tissue engineered construct succeeded in increasing the survival and proliferation of human articular chondrocytes [319]. Additionally, studies of a co-culture with human osteoprogenitor cells and HUVECs demonstrated an up-regulation of VEGF leading to the stimulation of angiogenesis [320]. Borselli *et al*. [321] were able to engineer a method of delivering constant low dosage VEGF release over several weeks and demonstrated that this increased the angiogenic response *in vitro* and *in vivo*.

With regards to bone formation (not just angiogenic capacity), hBMSC-seeded PLA scaffolds demonstrated increased bone regenerative properties in a femur segmental defect model [37]. Studies in which human osteoprogenitor cells were transformed to overexpress VEGF and then seeded on 3D scaffolds and hydrogels resulted in increased blood vessel formation through the scaffold [88, 93]. In a similar vein, Zhao *et al*. [322] placed a scaffold loaded with a plasmid for VEGF overexpression into rabbit bone defects and observed an increase in vasculature and bone healing in the

experimental group that was most transfected. Even without overexpression, culturing fibroblasts on a 3D structure instead of 2D monolayer has been found to result in dramatic increases in natural production of VEGF [323]. Demonstrating the interplay between osteoblasts and angiogenic cells, scientists have identified the expression of VEGFR1, VEGFR2 and other VEGF receptors on osteoblastic cells, increasing the known interactions between these cell types [297].

### Bone-Morphogenetic Protein -4

Over 20 different BMPs have been discovered to date [55]. Although this family of proteins is technically a sub-family of the TGF-β superfamily [16], it will be discussed separately due to its distinctive characteristics and properties of overt stimulation of osteogenesis. Different combinatorial expression of various BMPs has been shown in limb development (BMP 2, 4, and 7) [324, 325] and fracture healing [326]. Indeed, BMPs have been shown not always to form as homodimers but, in these situations, can also form heterodimeric complexes that may have increased bioactivity [324, 325]. BMPs are critical for the differentiation of osteoblast precursor cells during both osteogenesis and bone regeneration [230], and, when not signaling for such events, are stored in the skeletal matrix and osteoid [15]. The molecules signal through activation of the Smad pathway (particularly Smads 1, 5, and 8), which is a general pathway, but involves interactions with regulatory factors present in a cell-specific fashion, resulting in a gene regulation response that is tailored to the tissue [327]. BMP2 and BMP4 have also been shown to be stimulators of VEGF-mediated angiogenesis [328, 329], including an induction loop whereby VEGF stimulates endothelial cells to secrete BMP-2 [328]. The importance of BMP-4 is classically demonstrated through the knock-out mouse model, which dies in utero [330]. Studies of the role of BMP-4 in Xenopus have shown conclusively that BMP-4 plays a crucial role in the initiation of hematopoiesis. Moreover, studies on the unique process of distraction osteogenesis [331] have shown that BMP-4 is consistently expressed in the less differentiated cells [332]. Li *et al*. found

that the BMP-4 was mostly expressed during the lengthening and ossification stages and not during the consolidation phase (healing). As with the other growth factors discussed, BMP-4 has been shown to have a dose dependent response. The effect of dosage is shown in the following table [327]:

| | Low concentration | High concentration |
|---|---|---|
| BMP-2 | **No effect on expansion** | **Expansion ↓↓↓** |
| | **CFC output ↓** | **CFC output ↓↓↓** |
| | **Slight maintenance (25%) of SRC to day 6** | **Day-4 SRC ↓↓** |
| BMP-7 | **No effect on expansion** | **Expansion ↓↓↓** |
| | **No CFC output ↓** | **CFC output ↓↓↓** |
| | **No effect on day-4 SRC** | **Day-4 SRC ↓↓** |
| BMP-4 | **Complete differentiation to CD34 positive, CD38 positive cells** | **Maintenance of CD38 negative cells** |
| | **Expansion ↓↓** | **Expansion ↑** |
| | **CFC output ↓** | |
| | **Day-4 SRC ↓** | **Maintenance (83%) of SRC to day 6** |

**Table 5: Effect of BMPs on human hematopoietic progenitors maintained in *ex vivo* expansion cultures.** Low does is 5ng/mL, high dose is 25ng/mL for BMP-4 and 50ng/mL for BMP-2 and BMP-7. CFC = colony forming cell, SRC = combined-immunodeficiency-repopulating cell. Adapted from [327].

A significant amount of work has been carried out with the addition of BMPs to various scaffolds both *in vitro* and *in vivo*. However, some clinical trials of BMP application have been less successful [333, 334] than previous studies in animals [335-338]. Delivery of BMP-2 from a collagen sponge was shown to heal bony defects [339], and the use of such a scaffold has also been the subject of much study [316, 340]. The use of various synthetic scaffolds coupled with BMP-2 has also been investigated [55]; moreover, the US FDA has approved BMP products for bone regeneration; e.g., the InFUSETM bone graft (Medtronic Sofamor Danek, Memphis, TN, USA) [55]. Additionally,

much work has been done to transduce cells so that they over-express BMPs [341-344]. External delivery of FGF on various 3D scaffolds has also been demonstrated to produce osteogenic results [1, 38] and a combination of FGF and BMP has shown to be promising [38, 340, 345]. Li *et al*. [346] demonstrated that although VEGF and BMP-4 can act synergistically, they can also inhibit osteogenesis if applied in unfavorable dose and temporal distributions.

### Heparin

The pericellular matrix has been shown to have four primary functions: provision of a scaffold on which various types of cells can attach and spread; filtration and transport of proteins and nutrients both to and from a cell; modulation of cellular activity; and the binding of growth factors and cytokines to help create localized pools of these biomolecules [48]. Table 6 shows different glycosaminoglycans (GAGs) and proteoglycans (GAGs attached to protein cores), along with the proposed functions of their growth factor interactions [48].

| GAG/proteoglycan | Location | Function |
|---|---|---|
| **Hyaluronan** | **PCM** | **Intermediary protein that facilitates GF attachment to cell surface receptors** |
| **Heparin sulfate proteoglycans/heparin** | **PCM** | **Localizes GF to cell surface and increases likelihood that GF will bind to cell surface receptors. Forms complex with cell surface receptors and GF. Increases the lifetime of the ligand-cell surface receptor complex.** |
| | **Interstitial ECM** | **Localizes GF in ECM and creates a chemical gradient, thereby effecting cellular function** |

| GAG/proteoglycan | Location | Function |
|---|---|---|
| **Biglycan** | **PCM** | **Localizes GF close to cell surface** |
| **Decorin** | **Interstitial ECM** | **Sequesters GF away from cell surface and inhibits binding to GFR** |
| **Betaglycan** | **PCM** | **Forms complex with cell surface receptor and GF** |
| | **Interstitial ECM** | **Binds GF and may increase the half-life of GF. Sequesters GF away from the cell-surface and inhibits binding to GFR.** |
| **Versican** | **Interstitial ECM** | **Binds GF and chemokines** |

**Table 6: Various polysaccharides and proteoglycans which bind various growth factors (GF).** Figure adapted from [48].

Note, however, that such molecules do not homogenously surround the cell but are functionally distributed. Both heparin [230] and it's more ubiquitous related GAG, heparan sulfate (HS), are highly sulfated linear polysaccharides composed of repeating glucosamine-hexuronic acid disaccharide units [347]. Both show promise for applications in orthopedic tissue engineering as applied to fracture repair [38]. Heparin, a member of the HS family, has been shown to induce osteogenic differentiation [348, 349]. Heparin (as contrasted to HS) is only produced by connective tissue mast cells and not by osteoblasts or osteoclasts,. Although a component of the extracellular matrix and not in and of itself a growth factor, heparin will be discussed and experimented with in the context of growth factors because most known growth factors involved in osteoblastic cells are heparin-binding, e.g., FGFs, VEGF, TGF-β1, BMP-2, BMP-4, and IGF-II [90, 350].

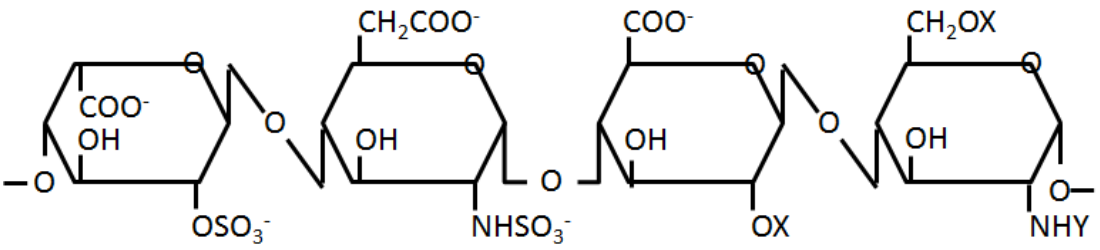



**Figure 18: Basic biochemical repeating structure of heparin.** Figure based on [230].

*In vivo*, HS GAG is a major component of bone ECM, and its basic structure is highly conserved between species (e.g., insects, mollusks, and mammals) suggesting that HS-ligand interactions are ancient and have evolved together. HS (which is slightly less sulfated than heparin) concentrates growth factors close to cells, protects them from extracellular proteases and facilitates binding to cell surface receptors [351]. The activities of HS have only recently been partially elucidated, although its ubiquitous presence on cell surfaces has been known for many years. HS polymers are localized to the cell surface through interactions with proteoglycans, especially syndecans and glypicans, the various isomers of which are localized spatially and temporally based on function, both normally and during the process of wound/fracture healing [351, 352]. HS binds to its wide variety of substrates with relatively high affinity ($K_d$ = 1-100nM) [351, 353] and helps to bring ligands and cell surface receptors together as well as targeting ligands to the cell for degradation (through stimulation of endocytosis) [354, 355]. Heparin, like HS, is capable of protecting growth factors from chemical and physiological degradation and sequestering depositories of growth factors [356-358]. Heparin has been shown to directly interact with endothelial cell growth factor (ECGF) in cell culture to increase the specific activity of ECGF as an endothelial cell mitogen, reducing cell doubling time and allowing the establishment of stable endothelial cell clones [359]. In addition to binding of factors, HS can be shed from the cell surface to form soluble proteoglycans [351]. The binding of heparin to growth factors is predominantly ionic in nature [360] and dependent on the positioning of basic amino

acids on the ligands in relation to the HS negatively charged sulfo and carboxyl groups [36, 230]. The biochemical process of HS synthesis is covered extensively elsewhere and will not be dealt with here [351], but has been demonstrated to enhance fracture repair through the growth factor interactions described above [361], and based on this finding we will be applying the related molecule heparin in our experiments. Heparin has been shown to act as a catalyst and change fibronectin conformation, leading to increased VEGF binding [352]. Furthermore, studies of heparin added to silk fibroin/collagen composite matrices [162] as well as PLA scaffolds [362] have demonstrated that heparin increases the mechanical strength of the scaffold, imparts a blood compatibility effect on the scaffold, and increases cell/scaffold interaction and adhesion. In addition to its growth factor interactions, heparin is well known, and used during surgery, for its anticoagulant properties, which, in the case of angiogenesis, prevent formation of thrombi in newly formed blood vessels [363].

As mentioned before, one simple approach to the use of growth factors to stimulate fracture repair is the injection of a solution of growth factor to the area where osteogenesis is desired. There are several problems with this approach including: a) growth factor delivered by injection would be rapidly cleared, b) growth factors have short half-lives, and c) even if applied in a high enough dosage to be initially effective, the active growth factor concentration in the area may not be present for a sufficient amount of time [4]. Heparin, it seems, is able to uniquely present potential solutions to improve upon this last issue: the delivery of growth factor in a timed-release particulate (often-times partly composed of HS or heparin) [364-367], or the delivery of growth factor on a 3D scaffold, e.g., of hydrogel or collagen. Studies have also been done of growth factor-release particulates being embedded in such three dimensional scaffolds [38, 230, 321, 367, 368]. In such a structure, one must check whether the particulate system causes a loss of heparin activity [230]. The formation of a 3D scaffold which binds HS leads to a postulated enhanced capability of the scaffold to both accept *ex-vivo*

applied growth factors as well as to interact with *in vivo* circulating signaling molecules and should lead to increased osteoprogenitor cell recruitment, differentiation, and proliferation [38]. Indeed, experiments investigating the addition of heparan sulfate to a synthetic scaffold *in vivo* have been attempted with just this reasoning in mind, although these studies were undermined by immune response to the polycaprolactone scaffolds [349]. A similar approach has also been taken in order to see if crosslinking of heparin to a number of materials could increase the efficacy of treatment either in and of itself or by increasing the efficacy of other conjugated growth factors [163, 237, 369-378].

# Chapter 2: Project Overview

In the Introduction, the reader was acquainted with the paradigm of the 3-component "jump-start" system of tissue engineering. That is, placing 3 critical components (scaffolds, cells, and growth factors) during transplant surgery into the patient or experimental model system in order to provide all the materials and signals necessary to stimulate osteogenic growth. The premise is that these components would fuse with the pre-existing bone and vasculature to create a viable, long-term solution to critical size bone defects. The following table demonstrates the overarching choices that governed the paradigm under which this DPhil project was undertaken.

| Our Model | Other options |
|---|---|
| Implanting all the components necessary to form tissue | Forming the tissue *in vitro*, then implanting |
| Transplant (requires surgery) | Injectable material |
| Natural materials | Synthetic materials |
| Constructed Scaffolds | De-cellularized scaffolds |
| Passive Culture | Biomechanical stimulation or bioreactor environment |

**Table 7: DPhil project paradigm.**

Consequently, the primary study, both *in vitro* and *in vivo*, examined and enhanced the interactions of materials, cells, and growth factors in order to create an optimized osteogenic scaffold through improved angiogenesis. As discussed in the Introduction, lack of sufficient angiogenesis is still one of the main challenges to tissue engineering, particularly for osteogenic applications. Thus, the overarching hypothesis of this thesis was that inducing better vasculogenesis and angiogenesis of the transplant would allow for more successful osteogenic and hematopoietic outcomes. This hypothesis was investigated from a number of different angles, each represented by a separate chapter in this thesis.

The first goal was to study the efficacy of various materials, which were made and modified by measuring their *in vivo* performance. Preliminary studies (presented in the transfer report and Appendix 4) were performed in bg/bg nu/nu xid/xid mice (NIH III nude mice) with these different materials either on their own, or in combination with human bone marrow stromal cells (hBMSCs) and/or human umbilical vein endothelial cells (HUVECs). Additionally, in support of a collaboration with Dr. Czernuszka's laboratory in the Materials Science Department at the University of Oxford, transplants of scaffolds containing specific channels designed to increase angiogenesis were performed. Transplants were judged by both histology and DAB chromogenic immunohistochemical staining for expression and presentation of α-smooth muscle actin (αSMA - which is found in blood vessel walls).

These preliminary experiments generated three general guiding findings for future experimentation. First, endothelial cells by themselves are incapable of forming vasculature because they are in and of themselves incapable of forming supporting structured tissue with extracellular matrix. Secondly, the inclusion of channels in the collagen transplant scaffold does not significantly increase angiogenesis of the scaffold as contrasted to the natural porosity of the scaffold with no added channels. Thirdly, the chosen material had a much larger effect on the success of the transplant than the crosslinking methods that were used. However, although these preliminary transplants provided guiding initial conclusions, the results are not presented here because of the fourth and most important finding: that in these exploratory experiments, the difference between the performance of scaffolds was not primarily due to scaffold material, cells, or channels, but rather due to which individual mouse in which they had been placed. These four findings are discussed further in Appendix 4.

The finding that the most significant factor was the host mouse was a watershed discovery, which brought to light issues with the laboratory model system used at the

time. Hitherto, the vast majority of ectopic bone formation studies have been performed in a particular strain of immunocompromised mice (the NIH III nude mice discussed above). These experiments included cells of either human or mouse origin, or no cells at all. Many labs across the world use this model, including the laboratory of the Skeletal Biology Section of the Craniofacial and Skeletal Diseases Branch of the National Institute of Dental and Craniofacial Research at the National Institutes of Health, Department of Health and Human Services (CSDB, NIH, DHHS). In this laboratory, ectopic bone formation with experimental materials, cells, and other various conditions had been studied in the same strain of NIH III nude beige mice since 1995.

However, it had become clear that the mice (which were obtained commercially and not congenic) had undergone genetic drift. This genetic drift was evidenced phenotypically by patchy black hair and gray skin that appeared in diverse patterns on these mice (which should have been hairless and beige) and by a marked inconsistency in the histology of transplants, including positive controls. In order to keep the project moving forward and until other immunocompromised mouse lines could be found and thoroughly tested for their bone formation performance, the possibility of developing a syngeneic transplant model for studying ectopic bone formation performance was investigated. The mouse that was chosen was the FVB-N/J mouse from Jackson Laboratories, discussed in more depth in Chapter 3.

In addition to developing a model in which ectopic bone formation could be studied in syngeneic immunocompetent mice, it was also necessary to create a protocol for the isolation of mouse endothelial cells. One of the main topics investigated in this DPhil project was the interaction of BMSCs and ECs, both *in vitro* and *in vivo*. In order to study the behavior of this co-culture in the syngeneic immunocompetent model, the endothelial cells would also have to be of FVB-N/J origin. The need to develop a new mouse model therefore led to a lengthy process in using the currently documented

methods for mouse EC isolation and devising a MACS protocol and FACS verification methodology.

*In vivo* studies of the performance of cells and materials are viewed as crucial, since *in vitro* assays are not necessarily a good measure of *in vivo* performance. For example, numerous studies have shown that given various stimuli, human BMSCs can differentiate into diverse cell types outside their lineage in ways that they have never been demonstrated to do in an *in vivo* situation. Nevertheless, *in vitro* studies are indispensable when taken in the context of *in vivo* studies, when not over-interpreted, and when performed with the proper controls.

There were two main topics that were investigated using *in vitro* techniques: a) the nature of the interactions between BMSCs and ECs, and b) methods that can be used to increase movement of ECs from the host into the scaffold in order to supplement the cells supplied in the transplant system. The former was tested by conducting a standard osteogenic assay, the protocol for which has been well-established in the research literature, and through measurement of growth factors secreted by the cells cultured together (using ELISAs). The latter was measured through 2D motility assays, which measure random movement in response to added cytokines, and through a Boyden chamber assay, which measures movement in response to secreted growth factors from BMSCs.

Thus, the aims of this project were to explore avenues to increase angiogenesis of a tissue engineered regenerative transplant system for bone, given the constraints provided by the paradigm.

To summarize, the over-arching hypothesis for this project can be broken down into several more specialized hypotheses which would be assayed through specific experimental methods:

- that syngeneic transplants in FVB-N/J mice would be sufficiently inbred as to form successful bone and hematopoiesis *in vivo* (syngeneic transplant from individual donors)
- that adding hydroxyapatite to a scaffold would increase the biomimetic nature of the scaffold, thus increasing bone formation and hematopoiesis *in vivo* (syngeneic transplant on different materials)
- that pooling cells from several donors prior to transplantation would be equivalent to the average results observed from individual donors *in vivo* (syngeneic transplant from either individual donors or pooled donors)
- that hBMSC and HUVEC interact and affect growth factor secretion (ELISA) and osteogenic differentiation abilities (osteogenic assay) *in vitro*
- that TGF-β, VEGF, BMP-4 and heparin could increase cell motility of HUVECs *in vitro* both in response to a non-directional stimulus (2D motility assay) and in response to a gradient (Boyden Chamber Assay) and stimulate their invasion into the transplant *in vivo* (DIVAA)

Accordingly, the objectives of this DPhil project which will be discussed in the final conclusion section were:

1. To demonstrate the viability of a syngeneic mouse model for bone formation
2. To study the *in vivo* performance of various collagen-based materials
3. To study the interaction of BMSCs and ECs cells in co-culture both *in vivo* and *in vitro*, and to measure the potential for stimulation of vasculogenesis
4. To study the effects of various growth factors on endothelial cell motility both *in vivo* and *in vitro*, to measure the potential for stimulation of angiogenesis

# Chapter 3: Can Bone be Made in Syngeneic Mice?

## Introduction

Prior to this project, the method for testing ectopic bone formation in mice had been conducted using immunocompromised mice, regardless of whether the seeded cells were of human or mouse origin. As it became evident that this model needed revision due to genetic drift in host mice, it was decided to attempt creation of a syngeneic model in order to test mouse cells. Since these mice were immunocompetent, it was therefore important to ensure that they were highly inbred, and for these purposes FVB-N/J mice were chosen. In this chapter we discuss the testing of bone marrow stromal cells (BMSCs) flushed from the long bones of these mice. BMSCs from a single mouse were generated, or were pooled from multiple mice for testing on various materials. These materials were made or modified in laboratories at the University of Oxford and the National Institutes of Health (NIH) and characterized through scanning electron microscopy (SEM). Our hypothesis was that it would be possible to create a syngeneic mouse model for heterotopic bone formation through use of cell-seeded collagen-based scaffolds.

## Methods

### Cell Culture

#### *Primary Derivation of mBMSCs*

Mice were euthanized via $CO_2$ inhalation followed by cervical dislocation, sterilized completely with one wash each of Wescodyne (an iodophor-based detergent disinfectant), water, 70% EtOH and water. The mice were then hand-dried with paper wipes and placed in the bio-safety hood. The hind legs were skinned and removed, taking care not to puncture the gut. The foot was also removed. In a dry petri dish, the femur and tibia were separated and cleaned of all surrounding muscle with a scalpel,

then placed in a dish containing a small volume of medium. Both ends of the bones were removed so that most of the growth plate was removed and the marrow was visible, while discarding as little marrow as possible. Using a 25-gauge needle and approximately 1 mL of BMSC medium per bone, the bone marrow was gently flushed into a conical tube and often emerged as a whole plug. When all legs were flushed, the solution was gently triturated about 10 times with a 10 mL pipette to create a single cell suspension. This cell suspension was then plated at a density of one T75 for the amount of bone marrow flushed from 4 legs (1 mouse). Half of the medium was changed after 1 week, and the cells were passaged after two weeks, at which point any remaining non-adherent cells were lost. The first few passages, in addition to trypsinizing the cells, required scraping in order to remove them from the flask surface, as they produced a large amount of ECM, while avoiding undue amounts of enzymatic processing.

### *Culture of BMSCs*

Bone marrow stromal cells were cultured in αMEM with the addition of 20% FBS, 1U/mL PenStrep, and 10mM Glutamax.

### *Freezing Cells*

Cells were frozen in a solution of 10% DMSO, 90% FBS and placed into a -80°C freezer in a Nalgene Cryo Freezing Container.

## Preparation of Materials

### *Making a Collagen Suspension*

1.5% w/v collagen type I in acetic acid was made and the pH was adjusted to 3.2. The solution was then homogenized in an ice bath using a hand-held blender for 30 seconds, followed by 30 seconds rest, three times. Air bubbles were removed by first placing under vacuum for 15 minutes, followed by centrifugation for 5 minutes at 5000 rpm.

### *Making Hydroxyapatite (HA)*

A solution of 0.166M calcium nitrate was added to an equal volume of 0.1M diammonium phosphate in a drop-wise fashion over the course of 3 hours while the solution was stirred and maintained at 50°C and pH 9-10. When the addition was finished, the solution and precipitate were left stirring for 12 hours at 50°C and then filtered using a funnel cup and filter paper. The HA collected on the filter paper was washed with deionized water, scraped in a sterile glass dish and heated in an oven at 80°C for 12 hours to obtain dry aggregates. These aggregates were then broken up through the use of a mortar and pestle.

### *Making a Collagen/HA Suspension*

A mixture of 3.5% w/v ground dry HA and 1.5% w/v collagen type I in acetic acid was made and the pH was adjusted to 3.2. The solution was then homogenized in an ice bath using a hand-held blender for 30 seconds, followed by 30 seconds rest, three times. Air bubbles were removed by first placing under vacuum for 15 minutes, followed by centrifugation for 5 minutes at 5000 rpm.

### *Making Collagen and Collagen/HA Scaffolds*

Collagen or collagen/HA aqueous solutions were placed in a PVC cylindrical mold (1 cm inner diameter, 1 cm height), covered on one end with PTFE (polytetrafluoroethylene) tape to provide a solid base and frozen at -20°C for a minimum of 8 hours. The ends of these scaffolds were then trimmed to remove any expansion out of the open side of the tube and to make all scaffolds uniform. Scaffolds were then transferred to a freeze dryer for a minimum of 8 hours. Scaffolds could then be removed from their molds and stored in airtight containers at room temperature.

### *DHT Crosslinking*

In order to crosslink scaffolds using dehydrothermal treatment (DHT), scaffolds were placed in a sterile glass petri dish in a drying oven and processed at less than

0.1mbar at 110°C for 72 hours. The oven was then cooled to room temperature, the vacuum released, and samples collected.

### *EDC-NHS Crosslinking*

In order to crosslink scaffolds using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide-N-hydroxysuccinimide (EDC-NHS), scaffolds were treated in an intermediate step after freezing and prior to lyophilization. After a minimum of 8 hours at -20°C the scaffolds were trimmed and PTFE tape removed. Scaffolds were transferred to 70% EtOH solution for a short time (less than a minute) to facilitate removal of mold. Scaffolds were mixed with a crosslinking solution consisting of 50 mM MES, 33 mM EDC, and 11 mM NHS in distilled $H_2O$, adjusted to pH 5.5. Scaffolds were then washed twice, for 1 hour each time, in 0.1 M $Na_2PO_4$ and once in distilled water. Scaffolds were then gently transferred back into the molds, frozen again at -20°C for a minimum of 8 hours, then lyophilized for a minimum of 8 hours and removed from their molds. The collagen scaffold synthesis and crosslinking processes, as well as stages where Gelfoam™ can be added for processing, are shown in Figure 19.

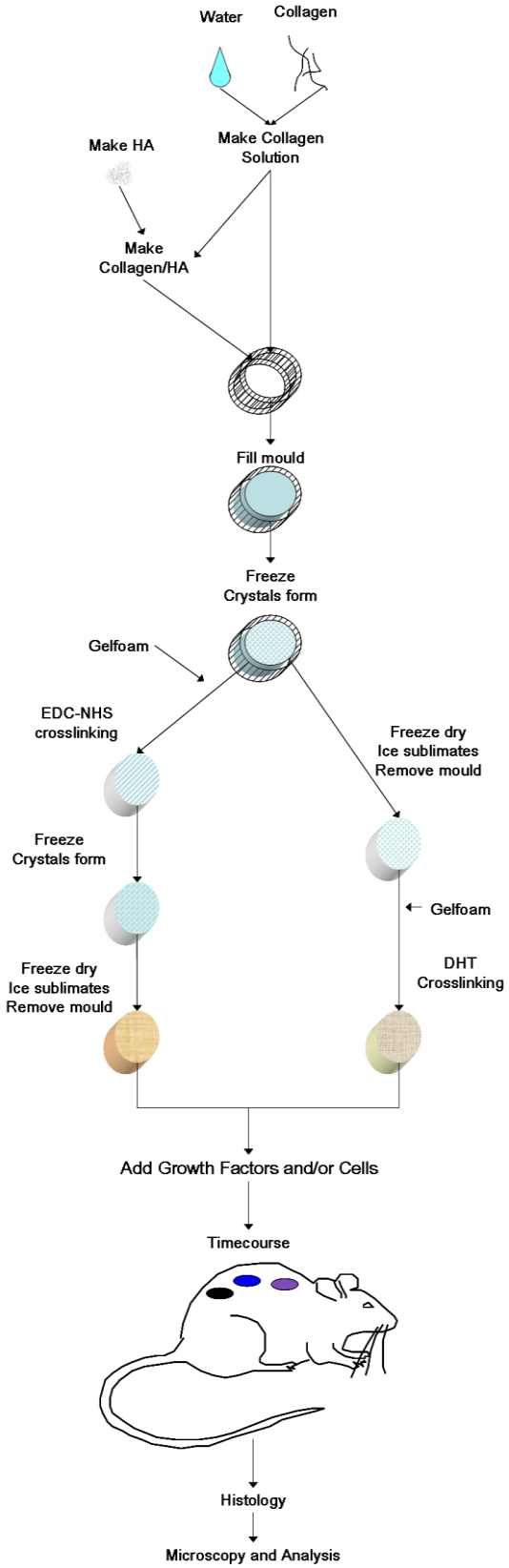



**Figure 19: Schematic of scaffold construction and modification prior to transplantation.** Figure adapted from [379].

### *Scanning Electron Microscope Characterization of Scaffolds*

Scaffolds were taken to New York University, where they were micrographed in collaboration with Professor Timothy Bromage. Both interior and exterior surfaces of the scaffolds were imaged to see if there was any difference. Micrographs were then analyzed using ImageJ to determine their pore size. Briefly, micrographs were opened in ImageJ and the scale was set using the scale bar from the scanning electron microscope (SEM) image (i.e., distance covered by pixels being related to real distance). The image was converted to a binary format and adjusted to maximize and clarify the difference between pores and pore walls. Pore size was then calculated using the ellipse model. This ImageJ analysis is shown below in Figure 20.

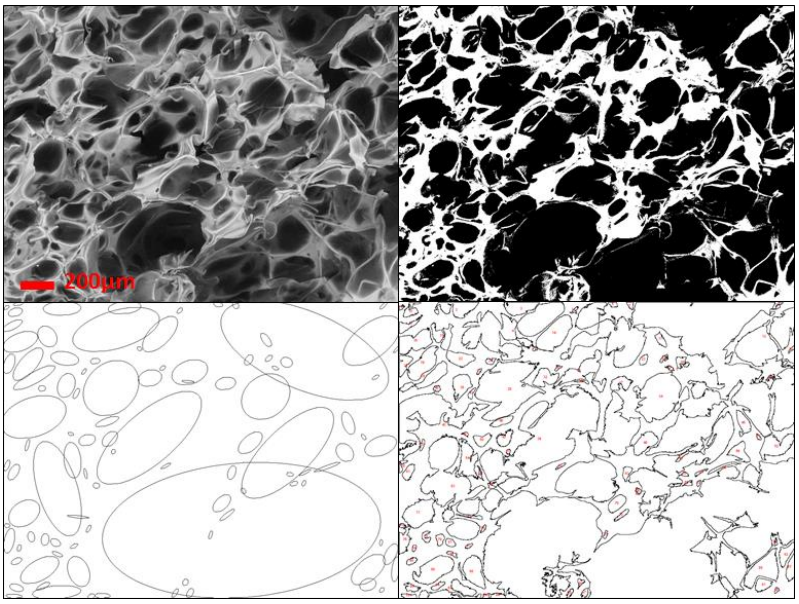



**Figure 20: How pore size is calculated.** Clockwise from top left: micrograph as taken, micrograph converted to binary and adjusted, the apertures detected by ImageJ, how ImageJ converts these openings into elliptical pores. The example shown is of Gelfoam™.

For porosity measurements, a grid with 100 interstices was superimposed on top of the micrograph and the number of interstices on pores versus pore walls was counted by eye. This method is demonstrated in Figure 21.

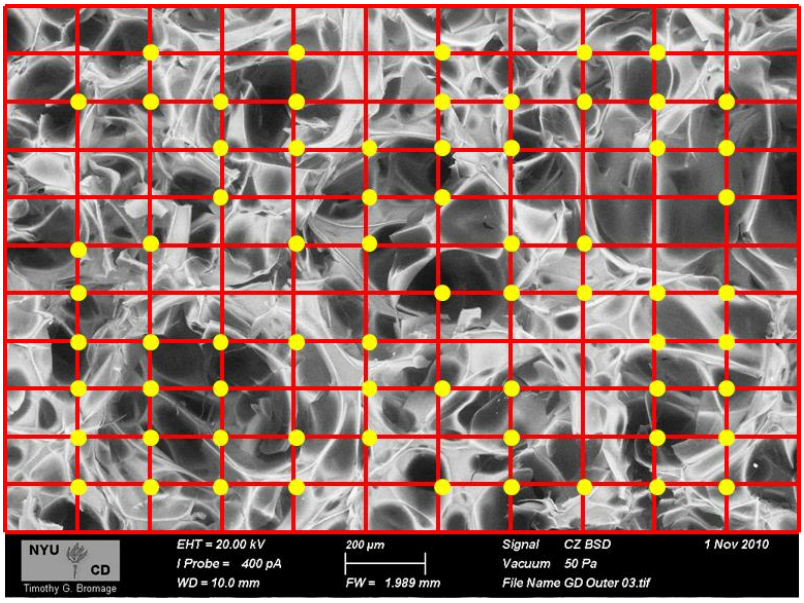



**Figure 21: Example of porosity measurement.** A grid with 100 inner interstices was overlaid on the SEM micrographs and the number of inner interstices that lie above a pore is counted by eye. This particular example is of Gelfoam™ crosslinked by DHT, measured to be 69% porous.

## Statistical Methods

It should be noted that all bar graphs presented in this thesis are the result of rigorous statistical analysis, performed with the assistance of GraphPad Prism. The statistical analysis was predominantly either one- or two-way ANOVA (analysis of variance), as appropriate for the data sample. Basic ANOVA tests the difference between two sets of data, assuming that the data sets have normal distribution and that the samples are not related to each other. This is measured by analyzing both the means of the sets of data as well as the variance within each set compared to the variance of the group. The post-tests applied to the ANOVA analyses were Bonferroni, with no matching. All error bars represent standard deviation *not* standard error. These post-tests are important to separate the individual differences between subsets of samples instead of the total variance of the set. With few exceptions, all data sets were compared with all other relevant data sets in the experiment; meaning that if indicator of significance (bar with * or multiples thereof) is present for an interaction, than the interaction did not reach $p < 0.05$ significance. Comparisons of data sets are qualified by

stars over several comparisons or a group of comparisons. For all interactions: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$

## Transplants

### *Seeding Cells on Scaffolds*

Scaffolds were soaked in αMEM and squeezed with forceps to remove any air bubbles. Meanwhile, cells were trypsinized, counted, centrifuged and resuspended to $2x10^6$ cells/mL. Cells were aliquoted in Eppendorf tubes at the desired concentration (generally $2x10^6$ cells per transplant), pelleted, and all but 30-50 μL of the medium removed. Cells were pipetted to uniformly distribute them in this small volume. Scaffolds were blotted on sterile Whatman filter paper to remove any liquid and then placed in the Eppendorf tubes to soak up the cell solution, drawing cells into the center of the scaffold. Immediately following cell seeding, scaffolds were taken to the NIDCR animal facility, and surgery was performed in accordance with Animal Care and Use Committee approved animal study protocol (ASP) #10-571.

### *Surgery Procedures*

Mice were anaesthetized using isoflurane gas, administered buprephenol to relieve pain, and ear punched for identification. After shaving or using depilatory to remove hair on the back of the mice (not necessary for nude mice), the mice were then swabbed first with iodine and then 70% ethanol to sterilize the area where the incision would be made. An approximately 1-1.5 cm incision was made along the center of the back using sharp scissors. Blunt scissors were used to form 4 subcutaneous pockets where transplants would be placed, and transplants were then placed using forceps. The wound was then closed through use of VetBond glue or secured with staples.

### *Harvesting Procedures*

Following the desired length of transplant time, mice were euthanized by $CO_2$ inhalation followed by cervical dislocation. The whole mouse was then perfusion fixed with 4% paraformaldehyde (PFA) and the scaffolds were subsequently removed. Scaffolds were fixed 18-24 hours in 4% PFA in PBS and then imaged using a Faxitron™ X-ray device to determine if decalcification was necessary. If indeed necessary, scaffolds were placed in 0.25 M EDTA in PBS solution, with a volume much higher than sample volume; e.g., 10 mL per small transplant, and the solution was changed every seven days, generally twice for a total of 14 days.

### *Histology*

When decalcified, transplants were placed in 70% EtOH and processed for paraffin embedding. Following embedding, samples were sectioned using a microtome to create 6 μm thick sections, mounted on glass slides (with 2-4 serial sections per slide), and dried at 37°C overnight.

### *Hematoxylin and Eosin Staining*

Slides were placed in a rotating dry oven at 50°C for 10 minutes to melt the paraffin and help the tissue to stick better to the slide. The hematoxylin and eosin (H&E) protocol is briefly described as follows: 2x5 minutes in xylene, 2x2 minutes in 100% ethanol (EtOH), 2x2 minutes in 95% EtOH, 3 minutes in tap water, 3 minutes in hematoxylin 2, 2x2 minutes in tap water, 1 minute in clarifier, 2x1 minutes in tap water, 1 minute in bluing reagent, 1 minute in tap water, 1 minute in 95% EtOH, 40 seconds in eosin, 2x10 dips in 95% EtOH, 2 minutes in 100% EtOH, 2x3 minutes in xylene. Slides were then mounted with a coverslip using Acrymount (Anapath).

### *Grading of Bone and Hematopoiesis from H&E Histology*

Bone and hematopoiesis of all samples stained with H&E was graded by three "blinded" researchers experienced in bone histology using a previously established

[380] 0-4 grading system (integers only). Following grading, results were compiled and exported to GraphPad for statistical analysis. The point scale used was as follows:

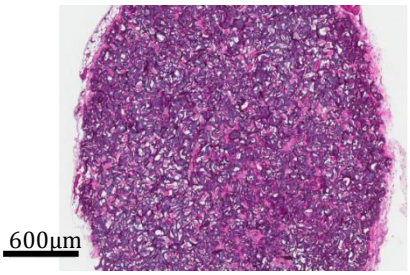
Bone Formation:

0) No bone formation
1) Minimal bone formation, just a single or a few bone trabeculae in one or a few sections
2) Low bone formation, multiple bone trabeculae are in several parts of some sections, but bone only occupies a small portion of the sections
3) Moderate bone formation, bone occupies a significant portion but less than one half of most sections
4) Abundant bone formation, bone occupies greater than one half of each section

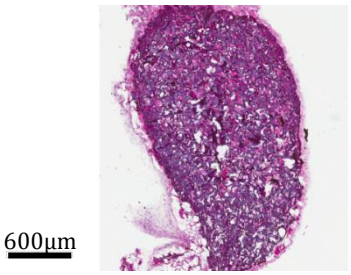
Hematopoiesis:

0) No hematopoiesis
1) Minimal hematopoiesis, just a single or a few small areas in one or a few sections
2) Low hematopoiesis, hematopoietic areas are in several parts of some sections but they occupy only a small portion of the sections
3) Moderate hematopoiesis, areas of hematopoiesis occupy a significant portion but less than one half of most sections
4) Abundant hematopoiesis, fields of hematopoiesis occupy greater than one half of each section

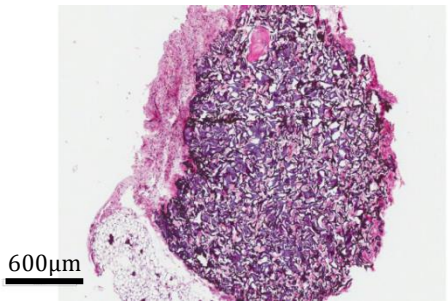
**Bone Formation 0**



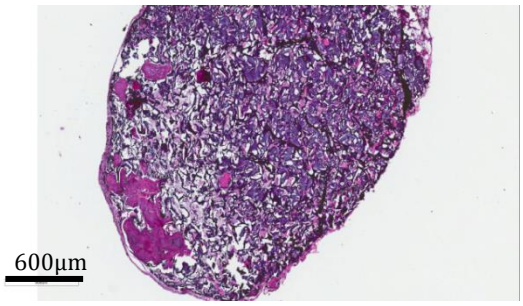

**Hematopoiesis 0**



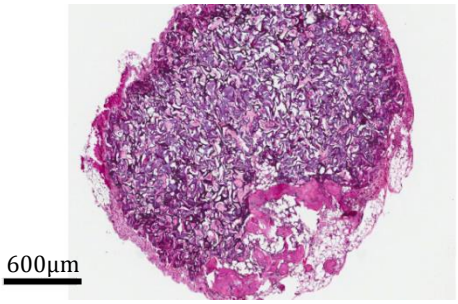
**Bone Formation 1**



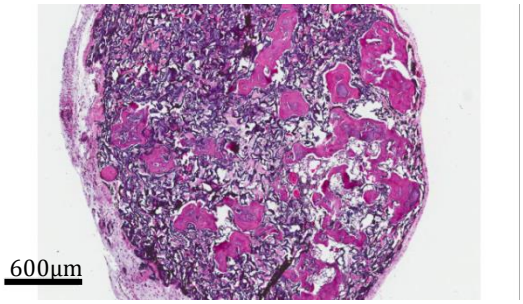

**Hematopoiesis 1**



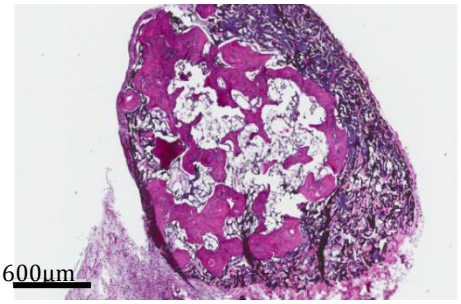

**Bone Formation 2**



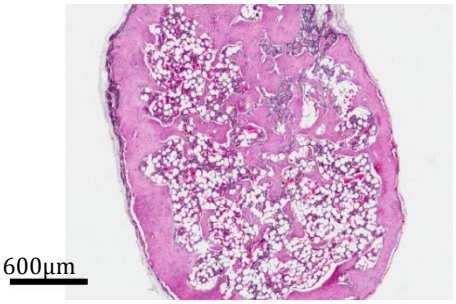

**Hematopoiesis 2**



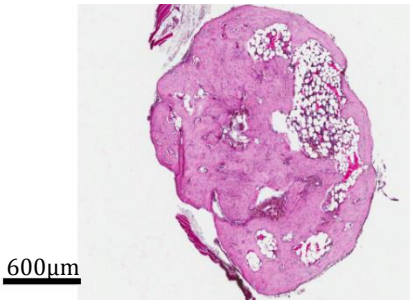

**Bone Formation 3**




**Hematopoiesis 3**




**Bone Formation 4**




**Hematopoiesis 4**

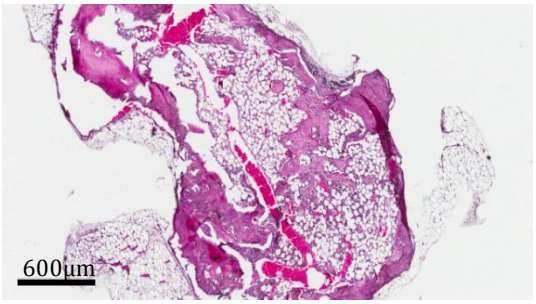



**Figure 22: Histological examples of each score for both bone and hematopoiesis** observed on Gelfoam™-based transplants stained with H&E.

## Results

In order to characterize the materials that were made (collagen fibril sponge and collagen fibril/hydroxyapatite composite sponge), modified (Gelfoam™ sponge), and purchased (Gelfoam™ sponge), samples were taken to New York University where they were imaged with Professor Timothy Bromage by SEM. The materials demonstrated a number of gross physical differences in structure, as shown below.

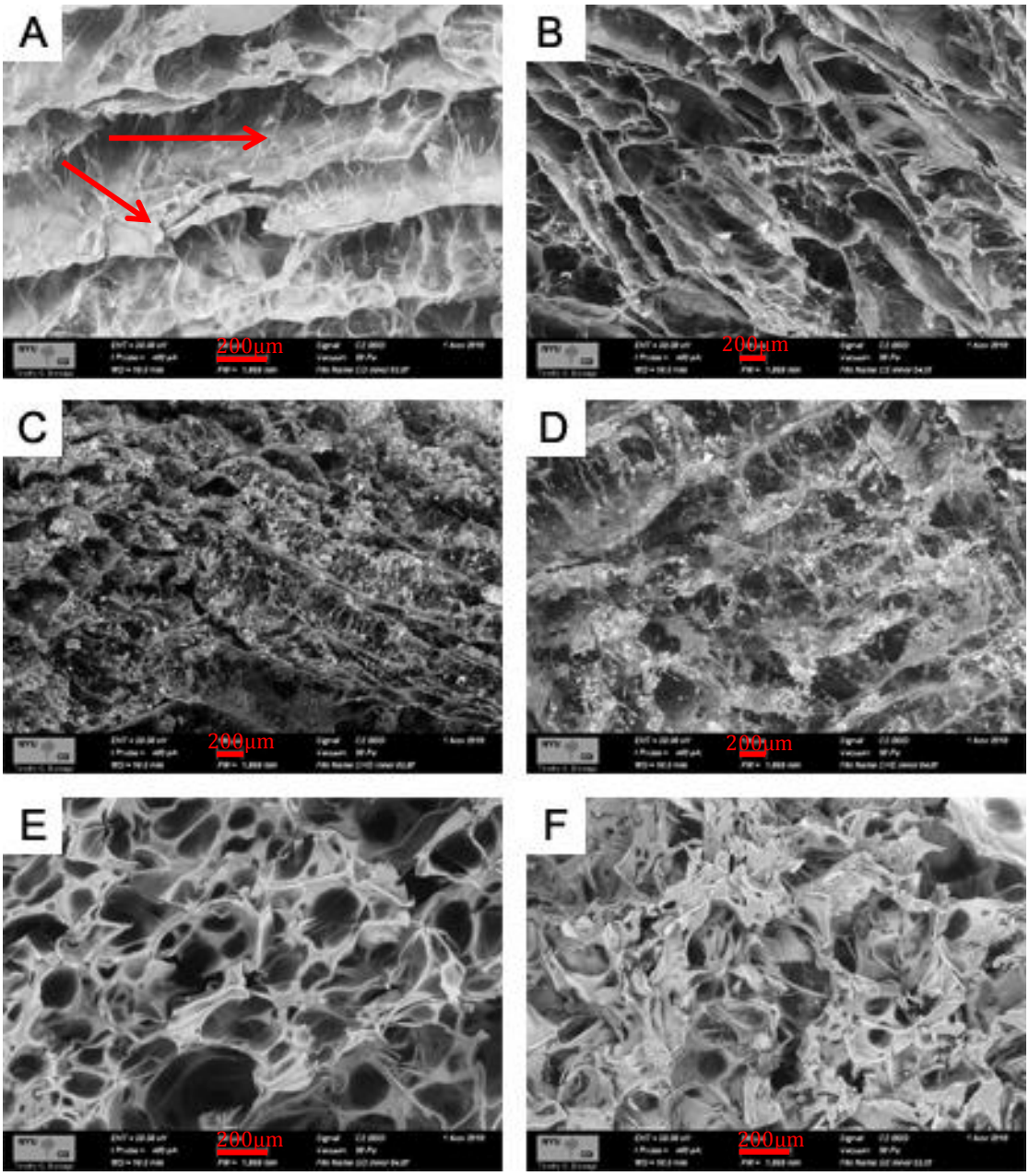



**Figure 23: SEM micrographs.** A) collagen crosslinked via DHT (arrows point to thick collagen walls and thinner connecting fibrils), B) collagen crosslinked via EDC-NHS, C) collagen-hydroxyapatite composite

crosslinked via DHT, D) collagen-hydroxyapatite composite crosslinked via EDC-NHS, E) Gelfoam™ crosslinked via DHT, and F) Gelfoam™ crosslinked via EDC-NHS. All images are at the same magnification.

Figure 23 demonstrates that there are major differences between the structures of the various scaffolds. Collagen fibril scaffolds appear to have larger pores with thick walls that are crisscrossed by thinner fibrillar structures (these two structures are shown by arrows in Figure 23A), which break up the geometry. On the other hand, Gelfoam™ walls appear to be more uniform throughout, and to have a more spherical pore structure, although there is still a range of pore sizes. The differences between DHT and EDC-NHS crosslinked collagen fibril scaffolds (A vs. B, C vs. D) are not particularly striking; however the EDC-NHS cross-linked scaffold does appear slightly denser. The differences between collagen and collagen/HA scaffolds (A vs. C) are apparent as granular HA particles are present throughout the composite scaffolds.

Interestingly, EDC-NHS crosslinked scaffolds appeared to have large amounts of residue on the collagen surfaces. This is also shown by comparison of A to B in Figure 23 above, where small particulates appear in the EDC-NHS crosslinked scaffold (B). Although the process was not supposed to leave any residue behind, just chemical facilitation of endogenous protein crosslinking, it appears that crosslinking chemicals are still present. In Figure 24 below, this residue is most apparent on the exterior surfaces, though to some extent on the interior surfaces (shown by arrows on Figure 24A and C respectively). Figure 24B, a very high magnification micrograph, demonstrates a type of crystal structure, which is unexpected from collagen alone.

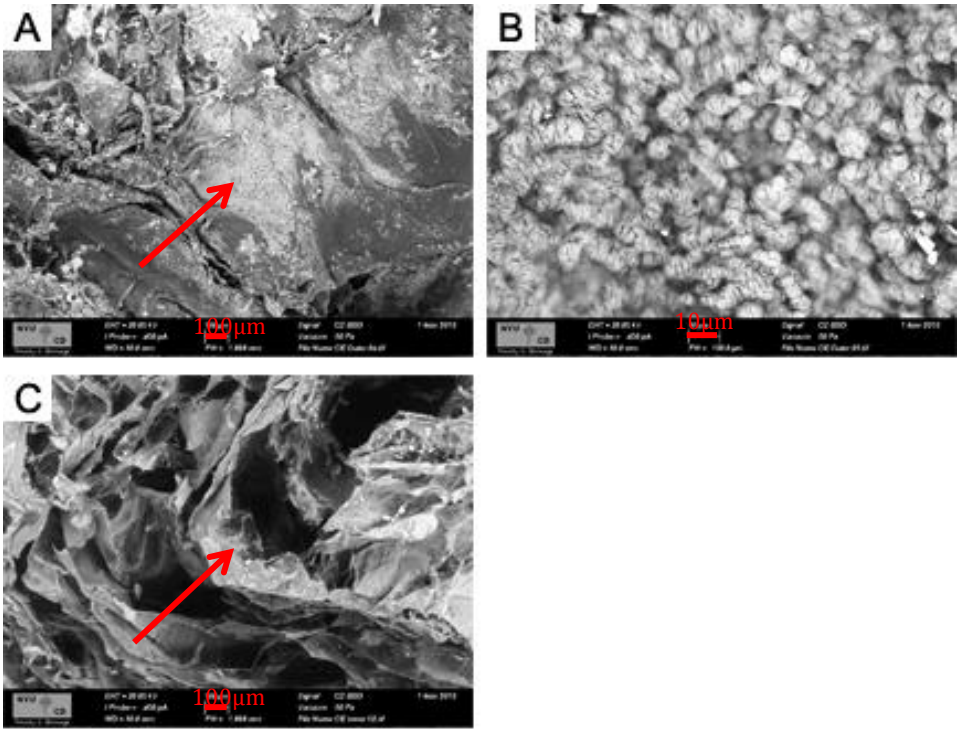
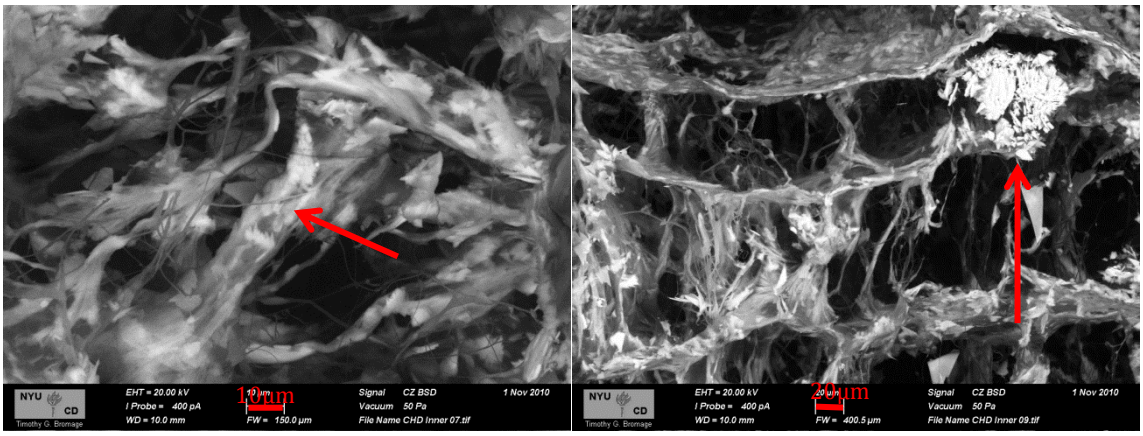



**Figure 24: SEM micrographs of residue** on the exterior surfaces of collagen scaffolds crosslinked by EDC-NHS (A and B) as well as on an interior cross-section (C). Arrows in A and C point to the residue discussed. B is a high magnification micrograph of the residue.

Hydroxyapatite appeared to be well mixed into the sponge and embedded in the scaffold walls. However, we also performed von Kossa staining of plastic non-decalcified scaffold sections, demonstrating that, while the HA was well incorporated where present, the HA was not evenly distributed throughout the scaffold.

A)



B)

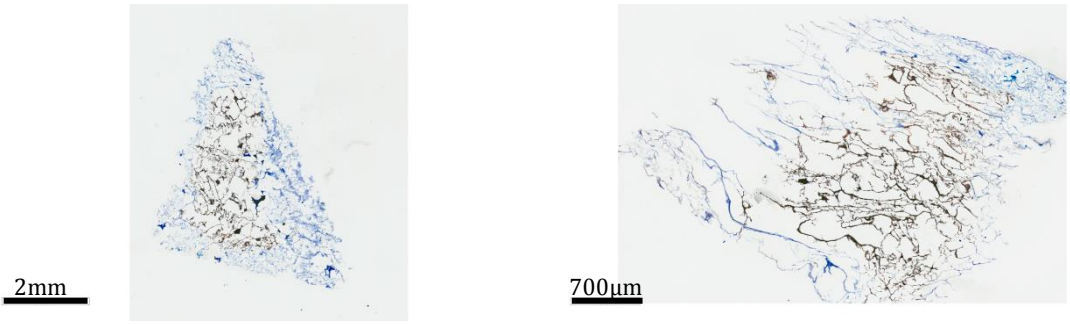



**Figure 25: A) High magnification micrographs of hydroxyapatite incorporation into the scaffold.** Arrows point to HA well integrated in the collagen (left) and to large chunks of HA embedded in the collagen (right). **B) Von Kossa staining of hydroxyapatite distribution in collagen/HA composite scaffolds.** Blue staining indicates collagen fibers, whereas black indicates the presence of calcium, in this case, HA.

Both Figure 21A and 21B demonstrate the nature of HA distribution within the scaffold. HA was shown to be concentrated towards the inner volume of the scaffold, virtually absent from the exterior. In areas where HA was present, the smaller crystals were well embedded in the scaffold (shown by an arrow in Figure 21A left). Larger blocks of HA were also found bound to collagen, but not completely surrounded by it (shown by an arrow in Figure 21A right). Figure 22 below shows HA presence in scaffold that had been transplanted *in vivo* and stained with H&E. In this image, the chunks of HA are clearly observed as blue crystal structures (shown by the arrow).

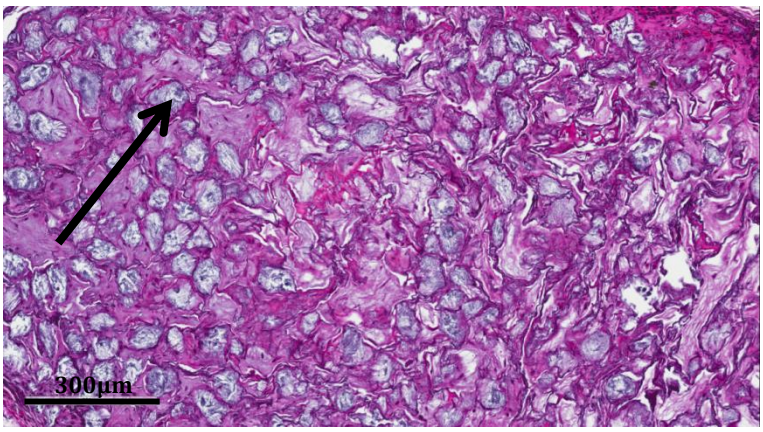



**Figure 26: H&E staining of a collagen/HA composite scaffold** seeded with hBMSCs and transplanted for 8 weeks in an NIH III Nude mouse. Image is at 10x magnification (scale bar is 300 µm). The arrow points to an example of an HA chunk shown by the granular blue structures.

Both the collagen and collagen/hydroxyapatite composite scaffolds were observed to exhibit significant morphological differences between their exterior surfaces and an interior cross-section. Collagen and collagen/hydroxyapatite scaffolds demonstrate almost wall-like structures on their exterior surface, shown by arrows (Figure 27 B, D, F, H). These collagen fibril-based scaffolds, however, possess the desired porous structure on their interior cross-sections (A, C, E, F). Gelfoam™ samples appear homogeneously porous throughout (I-L).

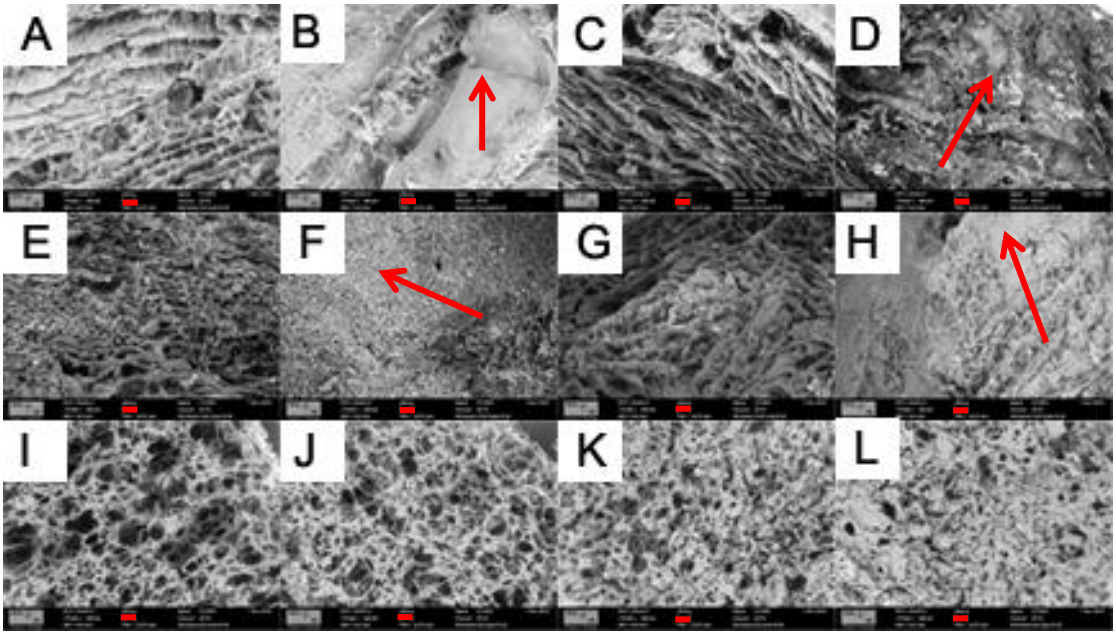



**Figure 27: Comparison of interior cross-sections versus exterior surfaces** for collagen crosslinked via DHT (A interior, B exterior), collagen crosslinked via EDC-NHA (C interior, D exterior), collagen-HA composite crosslinked via DHT (E interior, F exterior), collagen-HA composite crosslinked by EDC-NHS (G interior, H exterior), Gelfoam™ crosslinked by DHT (I interior, J exterior), and Gelfoam™ crosslinked by EDC-NHS (K interior, L exterior). The arrows in B, D, F, and H show the wall-like structures observed on the exterior surfaces of the synthesized collagen fibril scaffolds. Scale bars on all of these micrographs indicate 200 μm.

Pore size for the various materials was measured through micrograph image analysis using ImageJ software. The significant difference in the pore size and number between the exterior surface and interior cross-sections of the collagen and collagen/hydroxyapatite scaffolds during imaging was verified through quantification

shown below in Figure 28. For all scaffolds, the outside pore size is smaller than the interior pore size, though there appears to be no trend linking pore size and material or crosslinking method, demonstrated in Figure 29 (only collagen-DHT is significantly smaller than collagen/HA DHT).

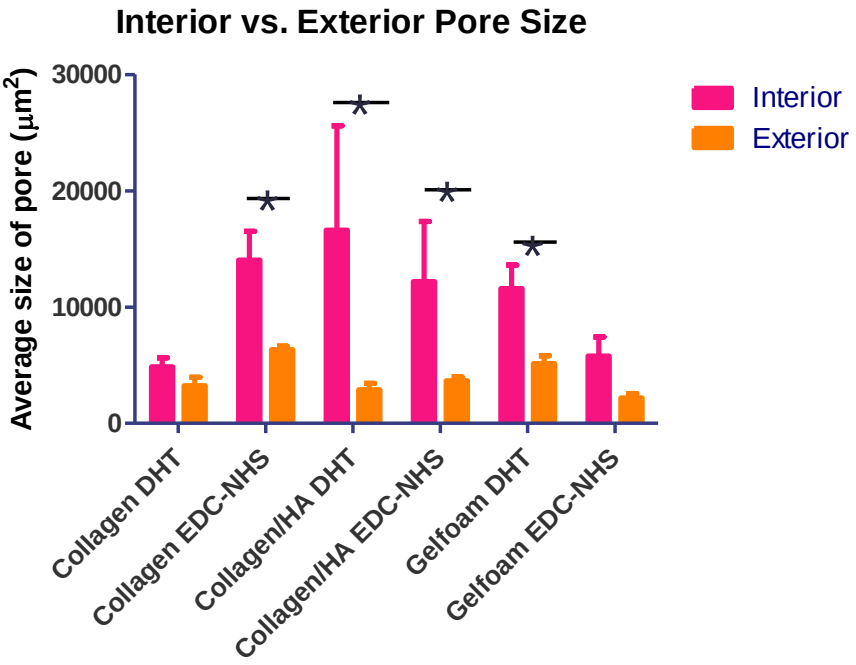



**Figure 28: Comparison of interior cross-section pore sizes imaged versus average pore sizes of exterior surfaces.** * = $p < 0.05$ n = 3 images per material type and location

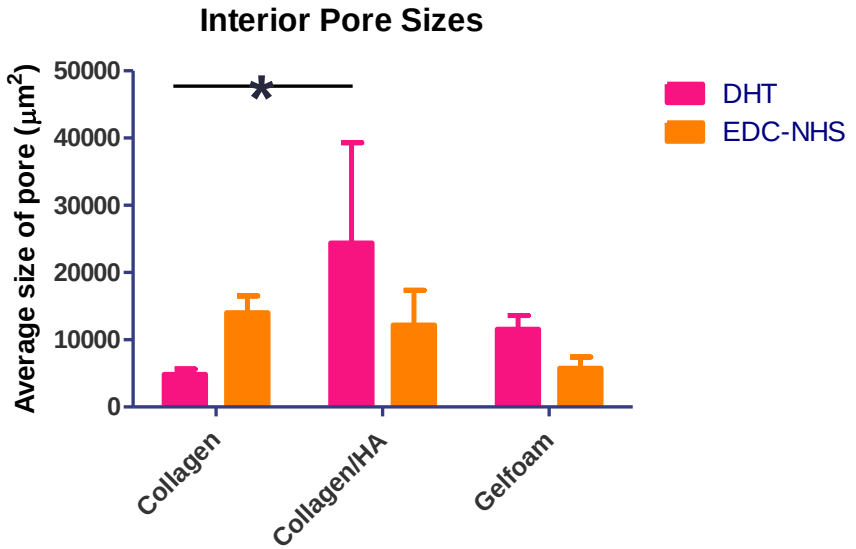
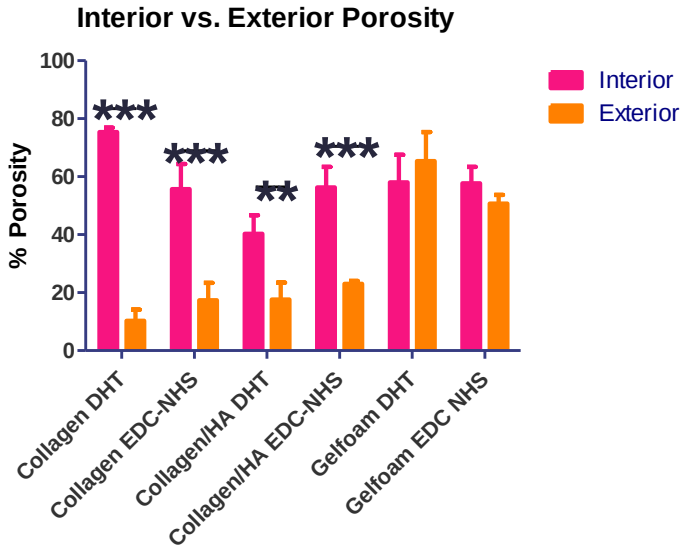



**Figure 28: Comparison of pore sizes** as varying by material and crosslinking method on interior cross-sections. * = $p < 0.05$ n = 3 images per material type and location

Porosity measurements, as measured through image analysis described in the methods section shows that Gelfoam™ scaffolds did not have significantly different porosity between interior and exterior surfaces, whereas porosity for all other scaffold materials and treatments were significantly reduced on their exterior surfaces (shown in Figure 30A). This is quantitative proof of the gross morphology observed above in Figure 27. Crosslinking method and materials did not change the porosity of interior cross-sections (shown in Figure 30 B).

**A**




**B**

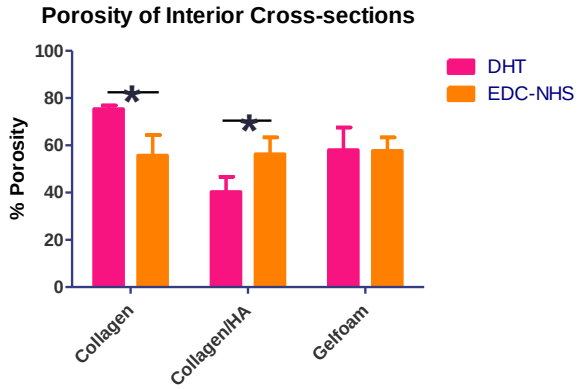



**Figure 30: A) Comparison of interior vs. exterior porosity across various materials. B) Comparison of crosslinking methods effect on porosity as measured by interior cross-sections.** * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ n = 3 images per material type and location

Subcutaneous transplants, as discussed in the Introduction, are a standard method of testing the efficacy of material and cell behavior *in vivo* [381]. Since 1995, the Skeletal Biology Section (CSDB, NIDCR at the NIH) had tested and found that the NIH III Nude beige mice from Charles River Laboratories were appropriate recipients for this experiment. However, starting in 2011, it became evident, by work performed by myself and colleagues in my laboratory, that this mouse strain was no longer ideal. Although immunodeficient, the strain is not congenic (they do not reproduce when congenic), and it was hypothesized that the mice were undergoing genetic drift. Genetic drift was demonstrated by the appearance of black patchy hair in varying amounts and patterns amongst the mice, which are supposed to be beige and hairless. Additionally, evidence of biological differences affecting experiments manifested when samples that had previously been shown to form bone, performed inconsistently and with much lower rates of bone formation. Therefore, the decision was made to use highly inbred mice to attempt the creation of bone using syngeneic cells. This experimental setup was also in direct response to a paper published in Nature Medicine that reported that an immune response in immunocompetent mice prevented bone formation from syngeneic BMSC transplantation in C57BL/6 mice [382]. In experiments performed here, FVB-N/J mice from Jackson Laboratories were used, which is an immunocompetent, certified inbred strain of haired mice.

The first syngeneic experiment consisted of mBMSCs from nine different FVB-N/J mice (4 bones harvested from each mouse), which were kept separate in culture. mBMSCs derived from each mouse were seeded into Gelfoam™ scaffolds, which were placed in 3 different mice. Transplants were harvested after 12 weeks.

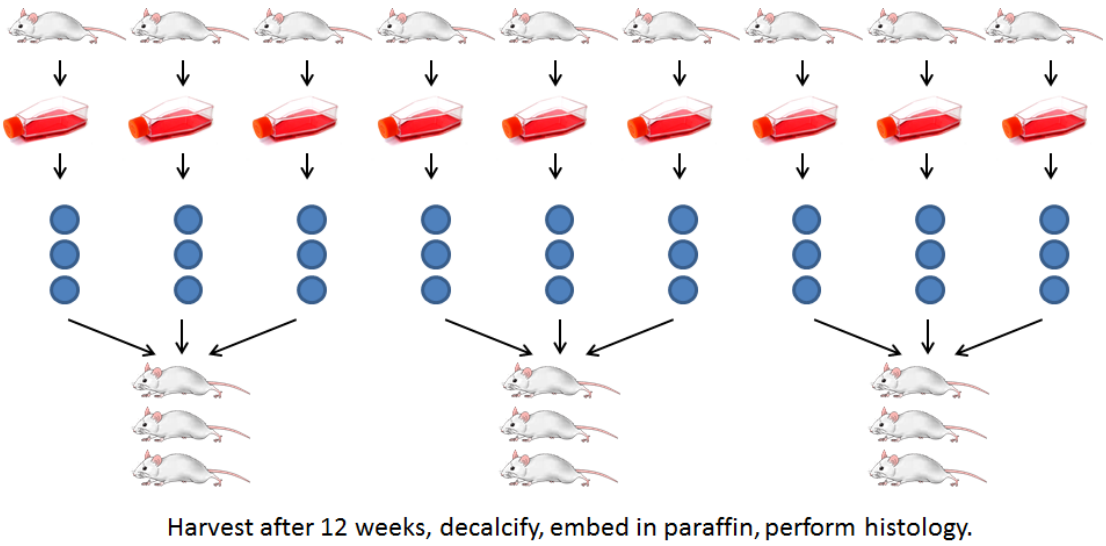



**Figure 31: Schematic of first FVB-N/J experimental design.** Note that one of each of the triplicate scaffolds is seeded in each mouse so that there are 4 different conditions per host mice (including the no cell control). The four transplants were rotated from one mouse to the next to negate any regional effects.

Following H&E histology, each transplant was graded for bone and hematopoiesis formation as described in the methods section.

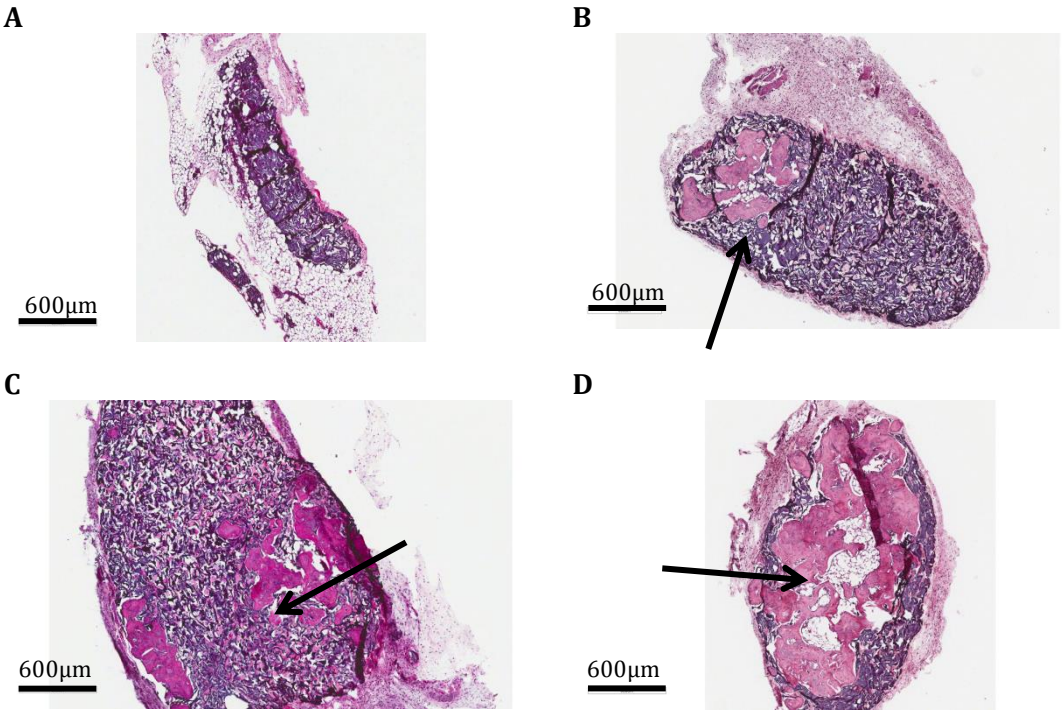



**Figure 32: H&E stained sections at 4x magnification from the initial FVB-N/J syngeneic transplant experiment.** A) An example of the no cell control. B, C, D) Examples of scaffolds seeded with mBMSCs from strains 2, 3, and 9, respectively. The arrows in B, C, and D highlight areas of osteoid tissue formation. The no cell control did not visibly produce osteoid tissue.

Figure 32 above demonstrates that scaffolds without any cells do not form a bone organ when transplanted ectopically in our syngeneic mouse model. In this instance, the term "syngeneic" has little meaning, as there are no cells of any sort on the transplant. This means that any tissue formed on the scaffold must be produced by cells that have migrated into the scaffold from the surrounding endogenous tissue. All Gelfoam™ scaffolds that had been seeded with syngeneic cells formed bone to a certain extent (shown by arrows). Some of these transplants also supported hematopoiesis, though to a lesser extent. The performance of these scaffolds with regards to bone and hematopoiesis is confirmed by the scores received by the grading panelists (Figure 33). While scaffolds differed in terms of performance, all seeded scaffolds made more bone than the no cells control, and although hematopoiesis also varied by strain, and scored less than bone, the results were also generally better than the negative control, which did not support hematopoiesis.

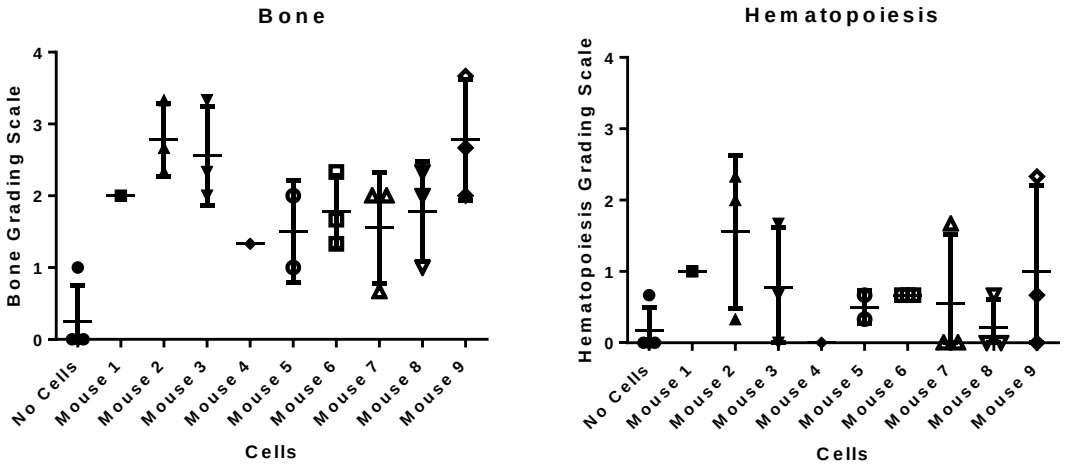



**Figure 33: Graded bone and hematopoiesis for transplants** made with cells from 9 separate mice on Gelfoam™ with a control of no cells. n = 3 transplants put in separate mice, except for cells from mice 1 and 4, where only 1 transplant of each was recovered

The second syngeneic experiment consisted of mBMSCs from five mice (4 bones from each mouse – these mice were not the same exact mice used in the previous FVB-N/J experiment), pooled immediately after flushing of the marrow, and expanded

together in culture for 3 passages. These cells were placed on four different materials, collagen crosslinked by DHT, collagen/hydroxyapatite composite crosslinked by DHT, Gelfoam™ crosslinked by DHT, and Gelfoam™ as purchased. Transplants were harvested after 12 weeks.

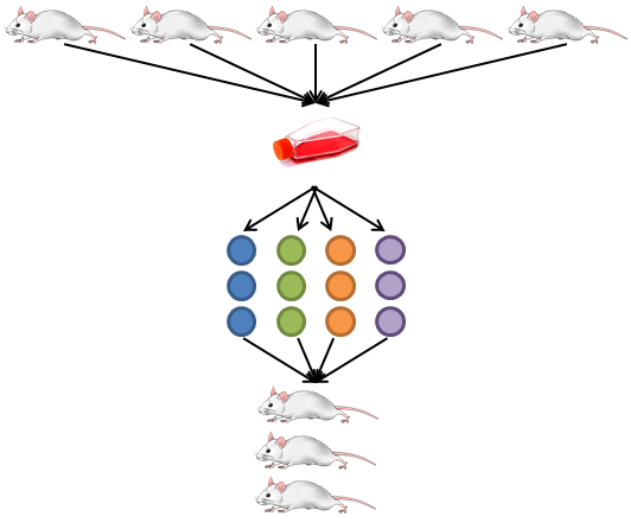



**Figure 34: Schematic of experimental setup for second FVB-N/J experiment.** The different colored scaffolds represent the 4 types of materials tested. Each mouse was transplanted with each of the 4 material types. The four transplants were rotated from one mouse to the next to negate any regional effects.

As with the first experiment, these scaffolds were graded for both bone formation and hematopoiesis.

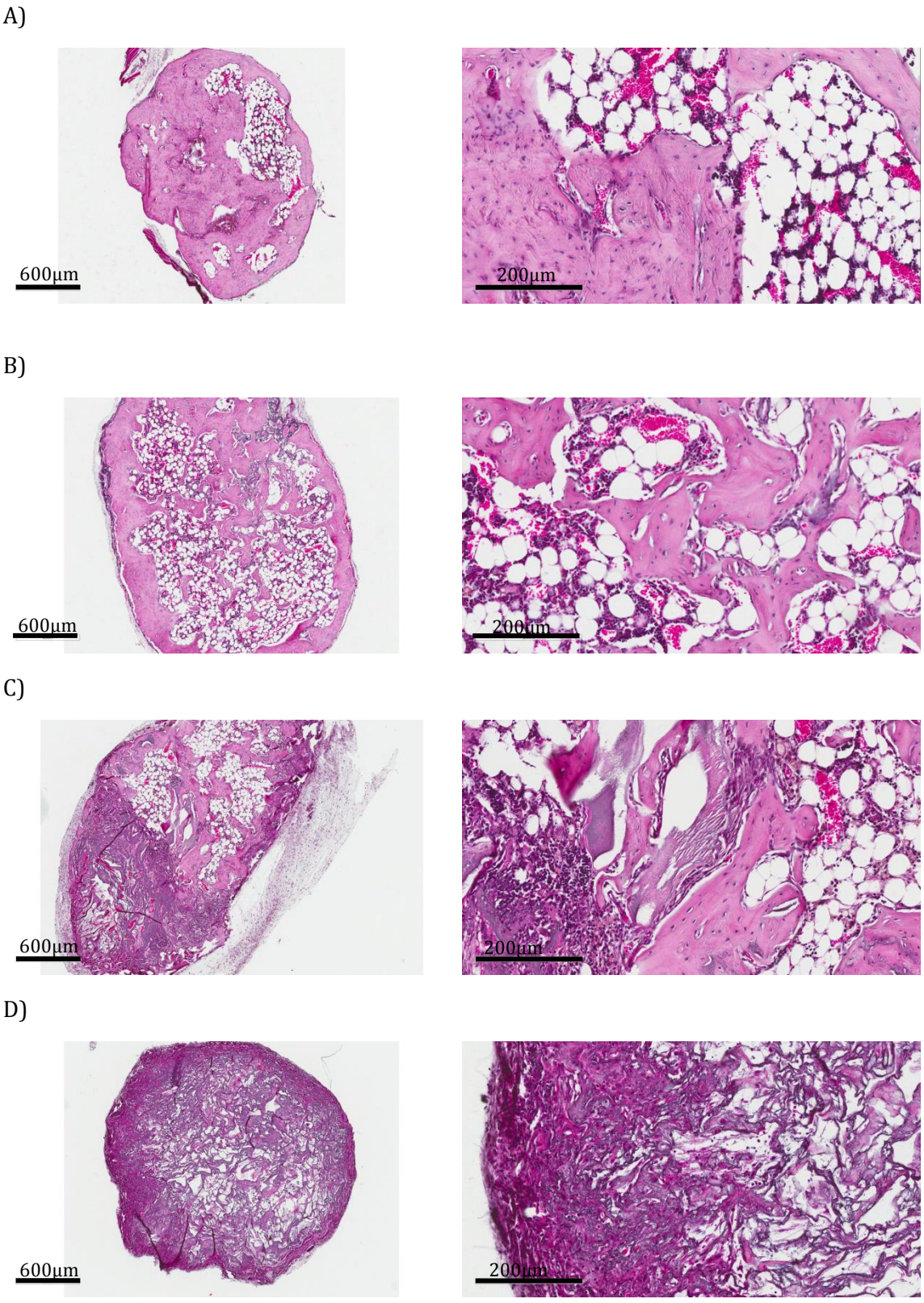
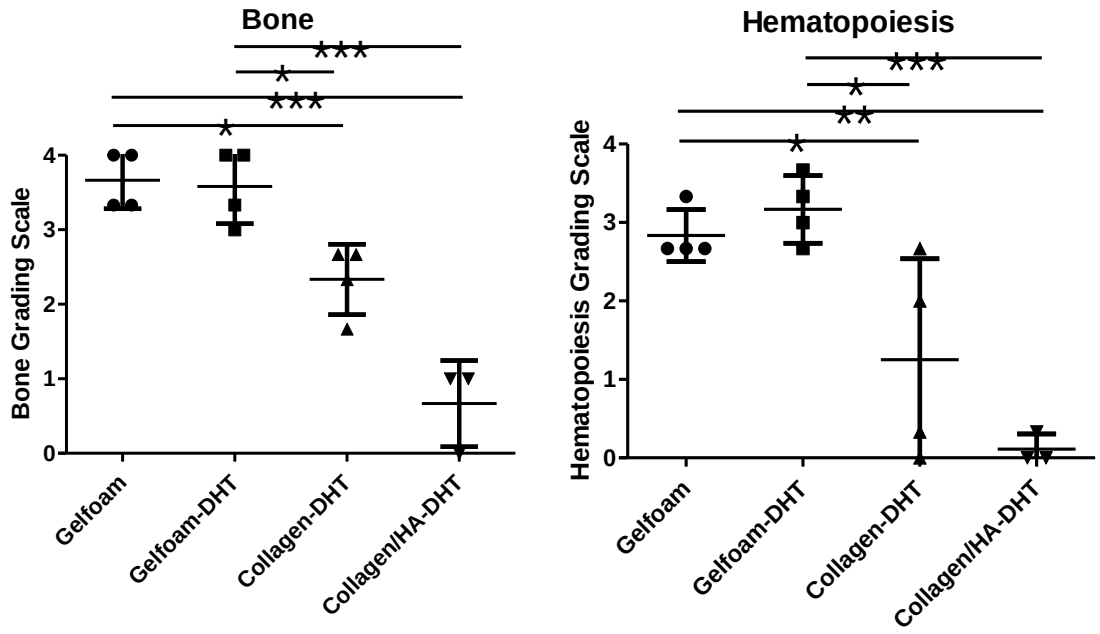



**Figure 35: H&E stained sections**, imaged at 4x magnification (left), and 20x magnification (right), **of pooled mBMSCs** on A) Gelfoam™, B) Gelfoam™-DHT, C) collagen-DHT, and D) collagen/HA-DHT. The arrows in A, B, and C highlight areas of osteoid tissue formation. The collagen/HA-DHT sample did not noticeably form osteoid tissue.

A




B

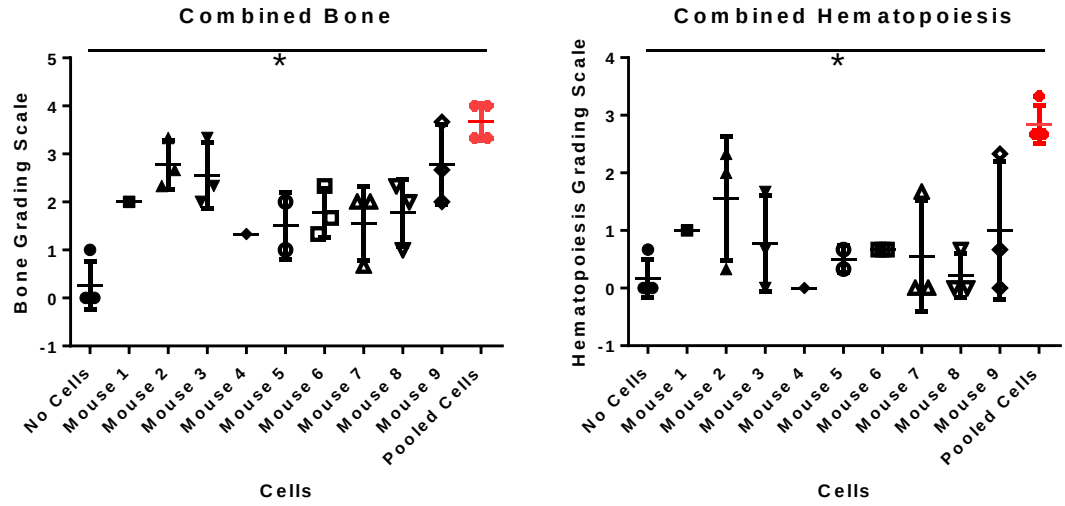



**Figure 36: A) Bone and hematopoiesis scores** for pooled FVB-N/J mBMSCs on a variety of materials. B) Comparison of pooled mBMSCs on Gelfoam™ shown in red, with a negative control of no cells and in comparison to the mBMSCs from individual mice from Figure 33. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ n = 3 transplants put in separate mice, except for cells from mice 1 and 4, where only 1 transplant of each was recovered

The results in Figures 35 and 36A demonstrate that the Gelfoam™-based scaffolds had both better bone and hematopoiesis as compared to collagen and collagen/HA composite scaffolds both visually as well as statistically when scored. In

particular, while there was no observable difference between Gelfoam™ and Gelfoam™-crosslinked scaffolds, there was a significant amount less bone and hematopoiesis formation in collagen fibril scaffolds, and even more so in collagen-HA composite scaffolds. There was a complete lack of osteogenic material in the collagen-HA scaffold. Observed histology consisted of densely packed cells towards the exterior of the scaffold and diminishing numbers of cells towards the interior of the scaffold. This phenomenon is most likely due to a combination of cell movement into the scaffold from the exterior of the transplant, as well as lack of cell survival in the non-vascularized center of the transplant. The pooled FVB-N/J mBMSCs on Gelfoam™ results can be compared to the mBMSCs derived from individual FVB-N/J mice from the first experiment (Figure 36B), and conclusively show that pooling cells from multiple mice results in consistently more bone and hematopoiesis in transplants.

## Discussion

Our SEM micrographs, as shown in Figure 27, demonstrate large differences between and within scaffolds. While the collagen scaffolds appear to have a wide range of pore sizes, the pores within Gelfoam™ scaffolds appear more uniform. Two other observations were also made by imaging of the scaffolds in the SEM. The first of these observations is the presence of a residue on the surface of scaffolds crosslinked by EDC-NHS. Although a chemical treatment, the EDC-NHS process, unlike some other chemical processes, does not incorporate any extra chemical molecules into the scaffold. Additionally, as the scaffold was rinsed several times after crosslinking and prior to re-freezing, the observed residue was wholly unexpected. The presence of the residue observed during SEM, as well as the lack of significant difference between DHT and EDC-NHS crosslinked scaffolds in preliminary transplant studies (discussed in Chapter 2), led us to concentrate solely on DHT crosslinked scaffolds for the remainder of the DPhil project.

The second observation was with regards to the HA incorporation into the scaffold. While in some parts of the scaffold, HA crystals were well incorporated in between collagen fibrils, in other places the HA appears to be present in larger pieces. This uneven distribution is most likely due to the decision to hand-grind the synthesized HA using mortar and pestle as contrasted to machine grinding. This decision was made based on previous work in the Materials Department at the University of Oxford, which indicated that the difference in results between hand-grinding and machine-grinding was largely insignificant, and that machine grinding resulted in more material waste. However, in this instance, the result was uneven, which was confirmed using plastic sectioning and von Kossa staining performed by a histologist, Helen Weimer, in our laboratory at the NIH. This staining method, in which collagen appears blue and calcified materials, in this case HA, appear black, confirmed the presence of HA being well incorporated, but with particles of different sizes present. Additionally, collagen/HA composite scaffolds demonstrated a lack of HA towards the edges of the scaffold. This observation is unexpected, as the scaffolds were made by placing a homogeneous aqueous mixture in a mold, freezing, and lyophilizing. As the mixture placed in the mold is homogeneous, this implies that one of two possible processes happens during freezing. Either the HA is pushed towards the center as the ice crystals form, or collagen travels towards the edges, and less-mobile HA is left behind. In any case, although collagen/HA composite scaffolds were tested in preliminary studies as well as for their performance in FVB-N/J mice; results from these studies, combined with the desire to use radiolucent materials in order to aid in live imaging by X-ray led to further study of this material being discontinued.

ImageJ analysis of the scaffold pore sizes, as shown in the results, showed no difference in pore sizes within each material treated with different crosslinking methods. The only significant difference in pore size was that collagen-DHT had smaller pore sizes than the composite collagen/HA-DHT scaffold. The most striking difference

was the difference between interior versus exterior pore sizes of most scaffolds (exterior pore sizes were smaller). In terms of the physiological relevance of this observation, an average BMSC is 25 μm in diameter, whereas a HUVEC is smaller at 17μm. Therefore, since the reduced pore sizes on the exterior of the scaffolds isn't necessarily a hindrance to cell migration; e.g. pore diameter decreases from an average of 134 μm to 90 μm for collagen EDC-NHS scaffolds, and from 125 μm to 68 μm for collagen/HA EDC-NHS scaffolds – both of these decreased pore sizes are still much larger than the diameter of the cells. However, sinusoids, the specialized blood vessel structures found in bone marrow, are approximately 100 μm in diameter, larger than these now reduced exterior pore sizes, but smaller than the interior pore sizes. This means that although cell migration throughout the scaffold may occur, the decrease in exterior pore size hinders vasculature bridging between the host and the transplant, regardless of lack of space restrictions on the interior *de novo* vasculogenesis of seeded cells.

Even more importantly than pore size was the decrease in porosity on exterior surfaces as contrasted to interior cross-sections. The calculation for freezing temperature, shown in the introduction (Figure 9), shows pore sizes, number of pores, etc. based on a set area. However, this equation assumes that the scaffold formed would be uniform, and our SEM analysis demonstrated that this homogeneity did not happen in the scaffolds made. If this equation had held, then the smaller exterior pore sizes would mean an increase in the number of pores in the same area. However, the decrease in pore sizes was actually indicative of the closing of pores and formation of a wall of collagen on the exterior of the scaffold. It can be hypothesized that this deviation from the ideal equation is due to the freezing process pushing a solid wall of collagen against the mold edge. The formation of these exterior walls resulted in a very significant and visible decrease in porosity on the exterior surface of all scaffolds made in this manner. However, the presence of this effect was not observed in the Gelfoam™ scaffolds

because they are fabricated as much larger sheets and then cut to size for commercial sale. These walls hinder not only the bridging of vasculature between the host and transplant, but also prevent the migration of cells between the two entities. The presence of these walls partially explains the unsuccessful preliminary tests with these materials discussed in Chapter 2 (and also in Appendix 4), which demonstrated no bone formation or hematopoiesis. However, for the purpose of all the experiments described hereafter, all tested collagen and collagen/HA scaffolds were trimmed of their exterior edges prior to transplantation based on the SEM findings.

Although a number of preliminary studies have been performed by other members of the NIDCR to try and determine cell markers that might predict bone and hematopoietic potential *in vitro*, no conclusive results have been reached. As discussed extensively in the Introduction, the only true way of testing the efficacy of a set of cells for development into specific tissues is to test via *in vivo* transplantation on a scaffold.

Due to problems the CSDB was finding in the typical mouse model (discussed in Chapter 2), it was decided to test the efficacy of a syngeneic mouse model. We first chose to test the performance of individual mouse BMSC cell populations, not arising from one cell (clonal), but the products of a bone marrow flush from one mouse each. This was to test for the variability of bone marrow-derived stromal cells from different mice, which differ just as cells from different patients' bone marrow demonstrate varied performance. In this case, bone marrow from 9 FVB-N/J mice, 4 legs per mouse, was flushed, cultured separately, seeded onto Gelfoam™, transplanted for 12 weeks, and analyzed for bone formation and hematopoiesis. As compared to a control of no cells in Gelfoam™, the all samples seeded with FVB-N/J BMSCs performed better. However, there was some variability between the performance between cells flushed from different mice, and, to a lesser extent from the same mouse, transplanted into different host mice. Both of these sources of variation are natural and expected. Although FVB-

N/J mice are inbred, and hence more similar than, for example, bone marrow cell populations derived from unrelated human patients, there will still be a natural variation in the bone forming potential, both in the capacity of bone marrow donor and transplant host. Note that all mouse donors were born on the same date, so age did not play a role. This variation might be the result of varying levels of hematopoietic cells within the populations, a known problem in dealing with mouse mBMSCs. Although the culture method used results in a dramatic decrease in the number of hematopoietic cells, the cell cultures were not specifically depleted of hematopoietic cells prior to seeding on the scaffold. Therefore, variations between cell populations from individual mice could impact on the level of osteogenesis and the ability to support hematopoiesis. The crucial message from this experiment is that FVB-N/J-derived BMSCs are capable of reproducibly forming bone and supporting hematopoiesis successfully in transplants in host FVB-N/J mice. This is a significant breakthrough in bone models in mice, as it was previously published that the immune system would inhibit or prevent bone formation of syngeneic transplants in any immunocompetent mice [382].

As we were successful in demonstrating that good bone performance could be found in scaffolds seeded with cells derived from individual mice, we hypothesized that pooling cells flushed from several mice together in culture would result in better histological results from the transplants, resulting in reduced variation between transplants than seen in the individual mBMSC cultures tested (Figure 36B). The additional advantage to pooling mouse cells is that it meant we could greatly decrease the number of transplants needed as we would effectively only be testing one cell population in a variety of materials.

Our results elucidate the performance, both of pooled cells as contrasted to cells from individual mice, and also of Gelfoam™ as contrasted to Gelfoam™-crosslinked by DHT, collagen crosslinked by DHT, and collagen/HA composite crosslinked by DHT. As a

material, Gelfoam™ demonstrated significantly improved bone formation and hematopoiesis over either collagen or the collagen/HA. The purpose of DHT was to increase the structural and chemical stability of the scaffold, but in this case, over a period of 12 weeks, it appeared to have no effect on Gelfoam™ scaffold performance when using mBMSCs. Impressively, the bone and hematopoiesis scores for the pooled cells were higher than any of the mBMSC populations from individual mice measured in the previous experiment. The better performance of pooled cells over cells from individual donors allows us to use pooled BMSC populations for future experiments, both *in vivo* and *in vitro*, reducing variability and increasing performance at the same time.

# Chapter 4: Gelfoam™ as a Transplant Material

## Introduction

As a follow-up to the experiments conducted in Chapter 3, it was decided to test the efficacy of Gelfoam™ and Gelfoam™-DHT scaffolds with either human or mouse bone marrow stromal cells (BMSCs). As human cells would be used, experiments were performed in our immunocompromised mouse model using NIH III Nude mice (a new congenic strain had not yet been identified). Previous experimentation by the Skeletal Biology Section had indicated that human cells degrade Gelfoam™ before bone had the opportunity to form. However, we hypothesized that DHT crosslinking of the Gelfoam™ scaffold provide chemical stability in the collagen that would delay its degradation. We were encouraged with regards to mBMSC performance on Gelfoam™ by our experiments in Chapter 3 in our syngeneic mouse model.

## Methods

### Primary Derivation of hBMSCs

The method for the primary derivation of hBMSCs is well described elsewhere [383]. Briefly, bone is obtained from aseptic surgical waste and cleaned of any associated tissue. The marrow is then scraped into αMEM and washed extensively to remove any remaining cells. Approximately 2 $cm^3$ sections of marrow are immediately placed in a tube containing αMEM supplemented with heparin to a final concentration of 100 U/mL. This suspension is centrifuged, resuspended in fresh medium, triturated through needles of decreasing diameter (up to 20 gauge) to break up aggregates, and passed through a cell strainer to yield a single cell suspension. Cells are then counted and plated on tissue culture plastic at approximately 5-10x$10^6$ cells per T75 flask in hBMSC medium. After one day of culture, the medium is replaced, and thereafter

changed approximately three times a week until approximately 70% confluent – then cultured as previously described for mBMSCs.

## Immunohistochemistry for α-Smooth Muscle Actin

Sections from transplants of mBMSCs and hBMSCs in Gelfoam™ or Gelfoam™ crosslinked by DHT were compared for the amount of blood vessel formation by staining for α-smooth muscle actin (αSMA), which is expressed on cells lining blood vessels. Slides were deparaffinized and hydrated through the process described at the beginning of the H&E staining procedure. Following hydration, slides were placed in a solution of 10 mM citric acid and microwaved for 2 minutes at 100% followed by 18 minutes at 20%, or, for more sensitive bone samples, for 10 minutes at 20% (antigen retrieval). Following microwaving, slides were returned to water and allowed to cool for 5 minutes. Slides were incubated in a dark moist histology chamber for 30 minutes with consecutive intervals in primary rabbit anti-αSMA antibody (1: 100 in antibody diluent) and also rabbit isotype control in antibody diluent (separated by a line from a hydrophobic-barrier pen). Following primary incubation, slides were rinsed 3 times for 2 minutes in PBS and then secondary antibody of Alexa Fluor 488 anti-rabbit IgG antibody was applied to both sections of the slide for 1 hour in the histology chamber. This processing was followed by 3 washes of 2 minutes each in PBS and then the slides were placed upright in a solution of 0.2% Sudan Black in 70% EtOH (dissolved under constant stirring for several hours and filtered just before use) for 30 minutes to quench the auto-fluorescence from bone. Following quenching, slides were rinsed three times in PBS and then mounted using aqueous fluorescence mounting medium containing DAPI (Soft-Set).

## Quantification of Fluorescent Staining Using ImageJ

Stained samples were imaged at 4x magnification for individual brightfield, DAPI, and GFP channels, as well as an overlay of all three. Brightfield (RGB) and GFP

images were overlaid using Photoshop and then the actual area of the transplants were cropped to avoid quantifying blood vessels from surrounding tissue included in the transplant section. The stained areas of these cropped transplants were calculated by ImageJ. Images were adjusted to minimize background and enhance signal - for the purpose of our 4x magnified transplants, a brightness and contrast range of 40-140. Once brightness and contrast had been optimized, the image was converted to binary format and pixels stained quantified as a percentage of total area. Output numbers were then imported into Excel and GraphPad for analysis.

## Results

Experiments were performed to analyze the difference between mBMSC and hBMSC in Gelfoam™ and Gelfoam™ crosslinked by DHT in NIH III nude mice. Transplants were harvested after 10 weeks. Samples were then graded for bone and hematopoiesis formation.

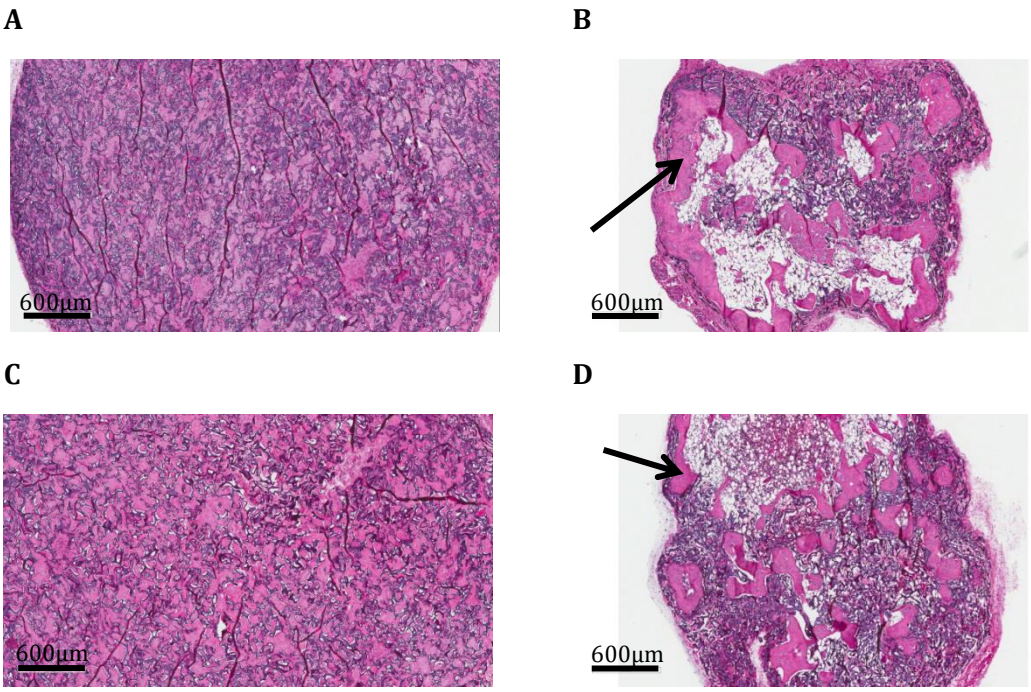



**Figure 37: H&E staining** of A) hBMSCs in Gelfoam™, B) mBMSCs in Gelfoam™, C) hBMSCs in Gelfoam™ crosslinked by DHT, and D) mBMSCs in Gelfoam™ crosslinked by DHT, all imaged at 4x magnification. The arrows in samples B and D highlight the formation of osteoid in these samples.

Figure 37 demonstrates clearly that human BMSCs (A and C) in Gelfoam™-based scaffolds did not form bone or support hematopoiesis, whereas mouse-derived BMSCs (B and D) performed well (arrows point at bone). There is almost no difference in performance between crosslinked and uncrosslinked scaffolds, aside from a higher variability in performance in DHT crosslinked scaffolds (demonstrated by the bar graphs in Figure 38). Of note, since the H&E sections in Figure 37 were imaged at the same magnification, and since scaffolds were all the same size, that there was significant contraction of the construct in the bone-forming mBMSC-seeded scaffolds.

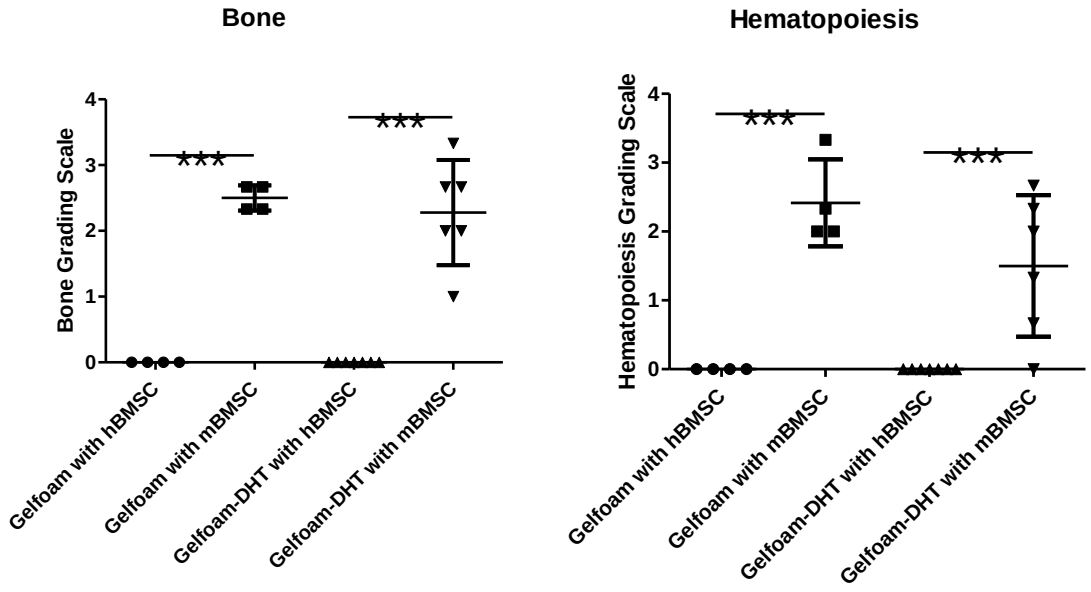



**Figure 38: Comparison of Gelfoam™ and Gelfoam™ crosslinked by DHT with either human BMSCs or mouse BMSCs**, graded for both bone and hematopoiesis. *** = $p < 0.001$ n = 6 transplants for each cell type on each material, however n = 4 for the non-crosslinked Gelfoam™, as only 4 of each were recovered

However, as shown below, the human BMSC transplants displayed an intriguing tissue histology.

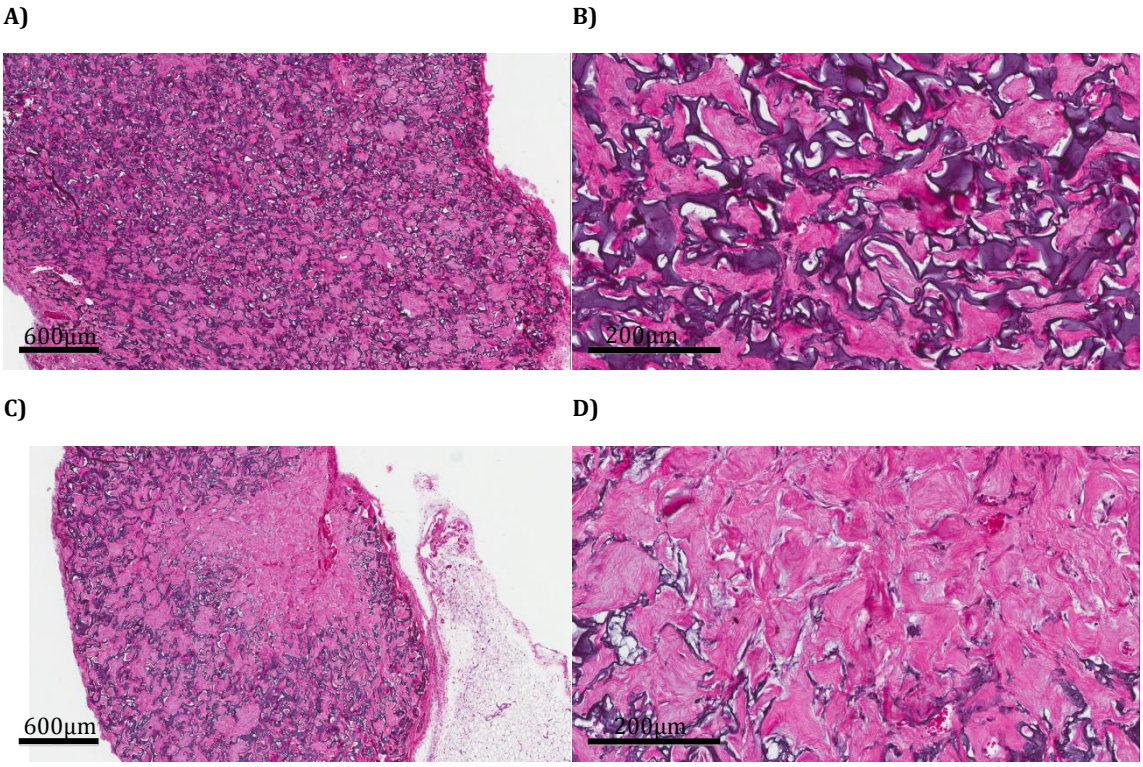



**Figure 39: H&E staining of human BMSCs** in A) Gelfoam™ and C) Gelfoam™-DHT, also in corresponding 20x magnification (B and D). The arrows in C and D highlight areas where the fibrous tissue had digested the Gelfoam™ to a large extent, and completely filled the space.

Many of the scaffolds resembled Figure 39A and B, with fibrous tissue spread throughout undigested scaffold, with very few visible cells. Interestingly, in some of the scaffolds, such as in C and D, the cells had completely digested the Gelfoam™ in certain areas and replaced it with only this filler matrix (shown by arrows).

In addition to H&E staining and scoring, these samples were immunostained for αSMA, a protein present in pericytes that line blood vessel cells, but otherwise absent from osteogenic tissue. These immunostained sections were imaged using fluorescence microscopy at low power magnification (4x). The images were then overlaid using Photoshop, and the resulting whole-section images analyzed using ImageJ. This method is shown step-by-step in Figure 40.

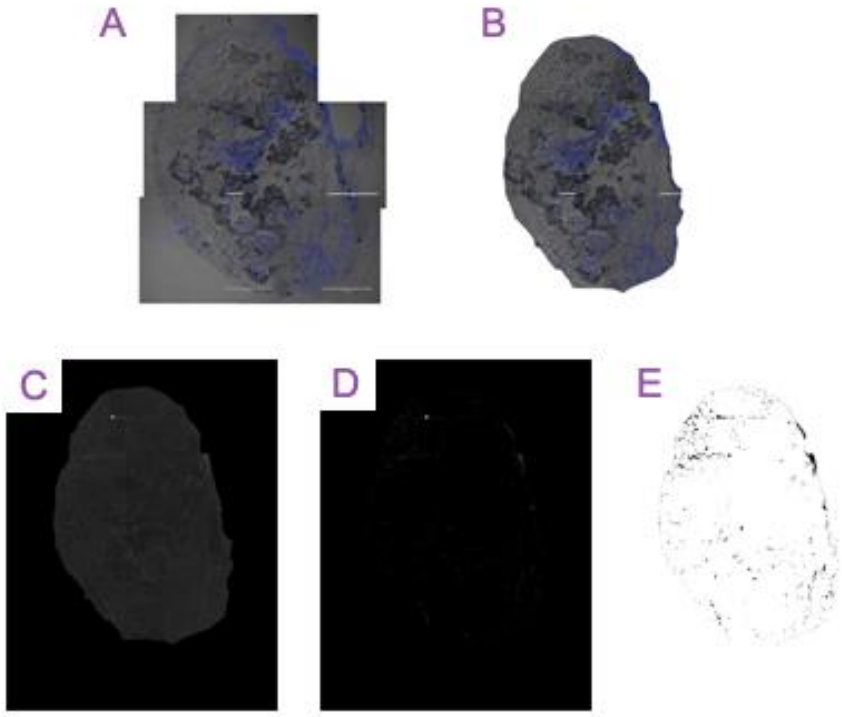



**Figure 40: The stages of IHC image quantification.** A) Placement of immunohistochemical microscopic images – overlay of brightfield view as well as DAPI and GFP channels. B) The transplant is cropped to ensure that the characteristics of the attached extraneous tissue are not quantified. C) The GFP channel transplant is isolated. D) Using ImageJ the brightness and contrast setting of the image are modified to minimize background and optimize signal. E) The image is converted to binary format and fluorescent pixels are quantified.

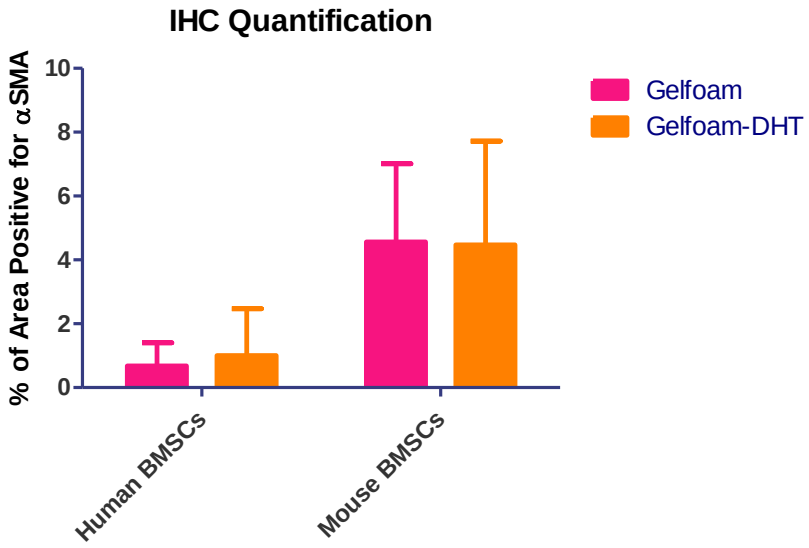



**Figure 41: Quantification of αSMA positive staining on Gelfoam™ and Gelfoam™-DHT scaffolds** seeded with either hBMSC or mBMSC. Although no data sets are significantly different from each other, 2 way ANOVA analysis demonstrates significant ($p < 0.05$) differences between human and mouse BMSCs. n = 4 for Gelfoam™ scaffolds and n = 6 for Gelfoam™-DHT scaffolds

Figure 41 demonstrates that there was significantly more αSMA expressed in mBMSC-seeded transplants as contrasted to hBMSC-seeded transplants. As αSMA expression is correlated to perivascular cells, we can conclude that there is a more extensive vasculature presented in mBMSC-seeded transplants. Higher vasculature is closely linked with osteogenesis and support of hematopoiesis, and thus our IHC quantification results were anticipated by the microscopy and scoring of such transplants is shown in Figures 37 and 38. Lower vasculature (αSMA) staining in hBMSC-seeded scaffolds was anticipated by the high magnification H&E histological analysis that demonstrated the lower number of cells in the fibrous tissue formed (Figure 39).

## Discussion

Clearly, since immunocompetent mice would have severe immunological responses to human cells, transplants with hBMSCs were performed in immunodeficient NIH III nude mice. Transplants were left in the mice for 10 weeks, then histologically analyzed and scored. The ability of the mBMSC-seeded Gelfoam™ to form both good quality bone and to support hematopoiesis (Figures 37 and 38) confirms our findings from previous studies that Gelfoam™ and mBMSCs are a good match.

With regards to the performance of hBMSCs in Gelfoam™, the only conclusion that can be reached from the results shown in Figures 37, 38, and 39 is that human BMSCs are incapable of forming bone when transplanted in Gelfoam™ (crosslinked and uncrosslinked) even in immunocompromised mice. This performance is not due to an immune response, as immune-related cells were not observed in the histological sections. The finding is also not due to innate inability of the host mouse to form bone, as the mBMSC scaffolds demonstrated. However, this result was not wholly unexpected, as previous results on Gelfoam™ with seeded hBMSCs in immunodeficient mice at the

Skeletal Biology Section laboratory had indicated that bone would not form as the human cells digested the scaffold too quickly, often resulting in complete absence of the scaffold at the harvesting time point (unpublished results). As all transplants were recovered, this is an improvement upon previous studies. However, we had hypothesized that DHT crosslinking would improve the characteristics of the scaffold enough to support osteogenesis. What we found was no observable difference between the performance of crosslinked and uncrosslinked Gelfoam™ scaffolds.

It is thought provoking to note that these hBMSC-seeded transplants, as harvested, were predominantly fibrous and acellular. The samples were stained with toluidine blue (not shown) but were definitely not cartilaginous (which would have appeared bright purple). Other knowledgeable researchers in the field have speculated that these samples, along with similar transplants observed in the past, may present pre-osteoid tissue, although generally by 12 weeks if the scaffold were to have become osteogenic it would have already done so. Although not understood, there appears to be a specific unknown difference between hBMSC and mBMSC interaction with the Gelfoam™ scaffold that results in a significant difference in histological outcome of cell-seeded transplants. Thus, we must conclude that, although Gelfoam™ showed promise in terms of murine tissue engineering, it fell short of the mark for clinical application of human tissue.

# Chapter 5: Does Combining Endothelial Cells (ECs) and Bone Marrow Stromal Cells (BMSCs) Increase Angiogenesis and Bone Formation?

## Introduction

In this chapter we investigate the effects of combined application of EC and BMSCs. The combination of these two cell types is logical, since both ECs and BMSCs play crucial roles in the healthy, natural process of bone formation. ECs, in particular, are necessary for the formation of a vasculature. However, preliminary studies discussed in Chapter 2 (and in Appendix 2) indicated that ECs, seeded into our fibrillar collagen scaffolds in the absence of any other cell type, were incapable of forming any tissue whatsoever and required the support of other cell types. This observed histology is due to the requirement for organizing cells (such as BMSCs or pericytes) that are not found in a pure EC culture [196]. Blood vessels require the physical support of a matrix (secreted by surrounding cells) as well as the chemical signals to maintain a stable structure, not to mention the cues needed to form the vascular network in the first place. Published studies on the combination of cell types have had mixed results [49, 224-226, 320] and we wished to test the nature of this interaction through both *in vitro* and *in vivo* studies. We hypothesized that the ECs and hBMSCs would have a positive effect on each other, and present advantages for tissue engineering over BMSCs or ECs alone.

## Methods

### Endothelial Cells

#### *Derivation and Culture of HUVECs*

Unlike our derived FVB-N/J-derived mouse ECs, discussed below, purchased human umbilical vein endothelial cells (HUVECs) adhere to gelatin-coated plastic, and

therefore were easier to expand *in vivo*. However, unlike BMSCs, which are very robust cells that can undergo multiple freeze/thaw cycles and stay viable for 5 days without media changes, HUVECs are trickier to culture. HUVEC growth in culture is very density-dependent. HUVECs do not thaw well. Additionally, HUVEC media is more complex and needs to be changed every 48 hours. Over the course of this DPhil project, four different sources of HUVECs were tested: Invitrogen (now Life Technologies), BD Biosciences, a collaborating lab (Dr. Giovanna Tosato at the NIH) that derived their own HUVECs from patient samples, and Lonza. All results presented in this thesis were obtained through use of Lonza HUVECs, except for the ELISA assay which was conducted with the primary cells derived by Dr. Tosato's lab (cultured in the same way as the Lonza cells).

HUVECs were purchased from Lonza and cultured on 0.2% gelatin-coated plates in M199 with the addition of 20% newborn calf serum, 5% human serum (heat-activated for 1 hour at 56°C), 1 U/mL PenStrep, and 10mM Glutamax, 25μg/mL ascorbic acid, 25 μg/mL heparin, and 15 μg/mL endothelial cell growth supplement (ECGS). Six hours after thawing and plating the cells, the medium was changed, and thereafter, medium was changed every 48 hours and cells passaged every 4 days. Cells were plated at a density of $0.7 \times 10^6$ cells/cm$^2$ and doubled in number approximately every 4 days.

### *Isolation of Primary Mouse Endothelial Cells*

From Lungs

Mice were euthanized and sterilized completely with one wash each of Wescodyne, water, 70% EtOH and water. The mice were then hand-dried and placed in the bio-safety hood. Sharp scissors were used to cut through the rib cage towards the head. After cutting through the diaphragm individual deflated lung lobes were removed and placed in cold αMEM in a conical tube. When all lobes had been collected the contents of the tube were transferred to a petri dish and all excess liquid aspirated, lungs were finely minced with sterile sharp scissors and transferred to a solution of

4mg/mL collagenase IV and 4mg/mL dispase in M199 medium in a 50 mL conical tube (5 mL per mouse). This tube of mouse lung-collagenase solution was placed on a rotator and incubated at 37°C for 45 minutes. The tissue solution was then spun down at 1500 rpm for 10 minutes and resuspended either in 0.5% BSA in PBS for magnetic activated cell sorting (MACS) or in EC medium for plating on gelatin-coated dishes. The cells extracted from all lobes averaged $8x10^6$ cells per mouse.

From Kidneys and Spleen

Mice were euthanized and sterilized as described above. Using sharp scissors, the mouse abdomen was opened to expose the peritoneal cavity. The spleen and kidneys were removed, taking care not to puncture the gut, and separately placed in ice-cold αMEM placed in cold αMEM in a 50 mL conical tube. When all organs had been collected the contents of the tubes were transferred to a petri dish and all excess liquid aspirated. The organs were finely minced with fresh sharp scissors and transferred to a solution of 4mg/mL collagenase IV and 4mg/mL dispase in a 50 mL conical (5 mL per mouse). This solution was placed on a rotator and incubated at 37°C for 45 minutes. The tissue solution was then spun down at 1500 rpm for 10 minutes and resuspended in 0.5% BSA in PBS for MACS.

From Bone Marrow

Mice were euthanized and bone marrow was flushed as described above. Once a single cell suspension was obtained, the solution was then spun down at 1500 rpm for 5 minutes and resuspended in 0.5% BSA in PBS for MACS. Total cells harvested from the bone marrow of 4 legs (1 mouse) averaged $51 \times 10^6$.

***MACS***

Cells were counted using a hemocytometer and resuspended at $10x10^6$ cells per mL in MACS buffer (0.5% BSA in PBS). Cells were then transferred to Eppendorf tubes, with a maximum of 1 mL per tube. Biotin-conjugated anti-mouse CD31 (an endothelial

cell marker) was then added at 10 µL/1x$10^6$ cells and allowed to incubate on a rotator for 30 minutes at room temperature. The cells were then washed three times with PBS and treated with desired secondary (either streptavidin-conjugated beads or anti-biotin-conjugated beads) also at 10 µL/1x$10^6$ cells for 15 minutes at 4°C. Meanwhile, a Miltenyi LS column was primed with 3 mL MACS buffer. Following incubation, the cells were combined with 1 mL MACS buffer, run through the column and cell fraction that passed through was collected; these are CD31 negative cells. The column was then washed twice with 3 mL MACS buffer. After placing a fresh collection tube under the column, 5 mL fresh MACS buffer was added to the column and the column was immediately removed from the magnet, and the fluid and column-attached cell fraction flushed using the plunger provided; these are CD31 positive cells. If desired, the negative cells could then be treated with another secondary antibody and run through another column as described, in order to increase cell yield. CD31 positive cells, CD31 negative cells, and unsorted cells were then tested for mCD31 using fluorescence-activated cell sorting (FACS) analysis.

### *FACS Analysis*

Cells were resuspended in 0.5% BSA in PBS and aliquoted to 1x$10^6$ per Eppendorf tube. Cells were then pelleted by spinning at 7000 rpm for 20 seconds and resuspended in 100 µL staining buffer, with or without 1 µL anti-mCD31-APC conjugated antibody. Cells were then incubated at room temperature in the dark for 30 minutes. Cells were washed 3 times with PBS, then resuspended in 200 µL Cytofix solution (1 part Cytofix to 3 parts PBS). Cells could either be analyzed immediately or kept, covered with tin foil, in the refrigerator. An Accuri-C6 flow cytometer analyzer was used to test cells.

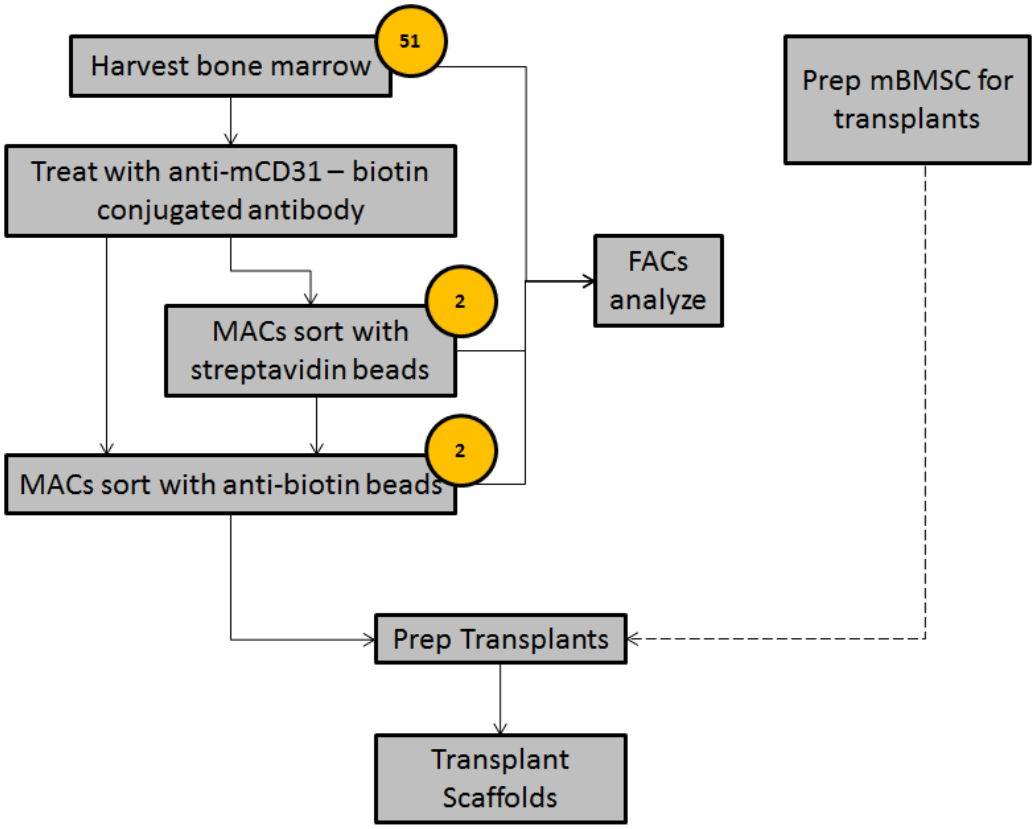



**Figure 42: Scheme for the isolation of mECs and combination with previously derived and cultured mBMSCs for transplantation.** Numbers in yellow circles indicate the number of cells x $10^6$ collected at each step from one mouse. Thus, approximately 8% of cells from the total harvest of bone marrow from 4 legs of an FVB-N/J mouse were actually found to be mECs.

## Osteogenic Assay

### *Experimental Setup*

$1x10^6$ cells were plated per well of a 6-well plate (coated with 0.1% gelatin), then, after 2 days, media was changed as desired and osteogenic factors added from a 10x stock solution. Final concentrations of osteogenic factors were: $5x10^{-3}$ M β-glycerol phosphate (β-GP), $1x10^{-4}$ M ascorbate-2-phosphate (AAP), and $1x10^{-8}$ M dexamethasone (Dex). Media and osteogenic factors were changed every 4 days. After 14 days, the culture was discontinued and samples were either stained for alizarin red S imaging and quantification or RNA collected.

### *Alizarin Red S Staining and Quantification*

When the time course was finished the medium was removed, cells washed with HBSS and fixed for 15 minutes in 4% PFA in PBS. Cells were then rinsed 3 times with distilled water, stained with alizarin red S (1% alizarin red S in 2% EtOH) for 20 minutes, then rinsed 3 times again. All fluid was aspirated and samples were left to dry at room temperature then stored at -20°C before imaging and quantification. In order to quantify the amount of alizarin red S in each well, the method was as follows. 2 mL 10% acetic acid was added to each well and incubated at room temperature with shaking for 30 minutes to detach the cells, which were then scraped, put in an Eppendorf tube, and vortexed vigorously to homogenize. The solution was then sealed and heated to 85°C for 10 minutes, cooled on ice for 5 minutes, and centrifuged at 20,000 x g for 15 minutes. After transferring 500 µL of the supernatant to a fresh tube, 200 µL 10% ammonium hydroxide was added to neutralize the pH to 4.1-4.5 and samples and standards were run in a 96 well plate on a spectrophotometer for $OD_{405}$.

### *qPCR Analysis of Osteogenic Assay Samples*

Osteogenic samples were produced in multiples of 6. When the time course was finished, 3 of the samples were fixed and stained for Alizarin Red, and the other 3 were lysed and processed for qPCR analysis. Cells were lysed using 350 µL cell lysis buffer (Buffer RLT) supplemented with 3.5 µL β-mercaptoethanol per well of a 6-well plate. Lysates were then scraped into Eppendorf tubes and vortexed until homogeneous. Samples were then transferred to QiaShredders and spun for 2 minutes at maximum speed. Samples were capped and frozen at -80°C until ready for processing. 350 µL 70% EtOH was added to the lysate, pipetted to mix, and added to an RNAeasy column from Qiagen (and processed using Qiagen reagents as described). Samples were spun for 15 seconds at 8000 x g and flow-through was discarded. 350 µL of RNA wash buffer (Buffer RW1) was added to the column spun for 15 seconds at 8000 x g and flow-

through discarded. 10µL DNaseI was added to 70 µL DNase-free/RNase-free buffer (Buffer RDD) per well and the mixed solution added directly to the column membranes and allowed to sit at room temperature for 15 minutes. Following this, 350 µL of Buffer RW1 was added to the column spun for 15 seconds at 8000 x g and flow-through discarded. The column was then washed twice with 500 µL prepared ethanol-based membrane-bound RNA wash buffer (Buffer RPE), first for 15 seconds and second for 2 minutes. The column was then carefully transferred to a new collection tube so as to not carry over any Buffer RPE. The column was then spun for 2 minutes to remove any remaining solution. Finally, the column was placed in a collection Eppendorf tube, 30 µL RNase-DNase-free water added, let to sit on the column membrane for 1 minute, and then spun for 1 minute at 8000 x g.

Following RNA extraction, the amount of RNA obtained was quantified using a Nanodrop spectrophotometer and the volume needed for 300 ng RNA calculated. Using these calculations, 300 ng of RNA was converted to cDNA with the iScript cDNA kit using the following reaction per sample: 1 µL transcriptase, 4 µL 5x iScript reaction mix (contains a mix of oligo(dT) and hexamer primers), the volume for 300 ng of RNA (calculated from Nanodrop determined concentrations), and the remainder PCR-grade water up to a total volume of 20µL.

For qPCR, cDNA was diluted 1: 100 from the stock and primers were diluted 1: 10. Primers were designed by Dr. Natasha Cherman of the Skeletal Biology Section, using Beacon Designer 6 software. The basic qPCR reaction consisted of 12.5 µL SYBR Green, 1 µL each of the forward and reverse primers of interest, 0.5 µL $H_2O$, and 10 µL of cDNA. The instrument used was a CFX-96 Real-Time PCR System paired with a C1000 Thermal Cycler. The protocol run was as follows: 95°C for 3 minutes, then 40 cycles of 95°C for 10 seconds, 62°C for 30 seconds, and 65°C for 5 seconds. Results were exported into Excel, and reformatted for input into GraphPad for statistical analysis.

## Enzyme Linked Immunosorbent Assay (ELISA)

ELISAs were carried out as per the instructions for each particular kit from R&D Systems, and using support reagents for ELISAs as sold by R&D. A diagram of how this assay works is shown below.

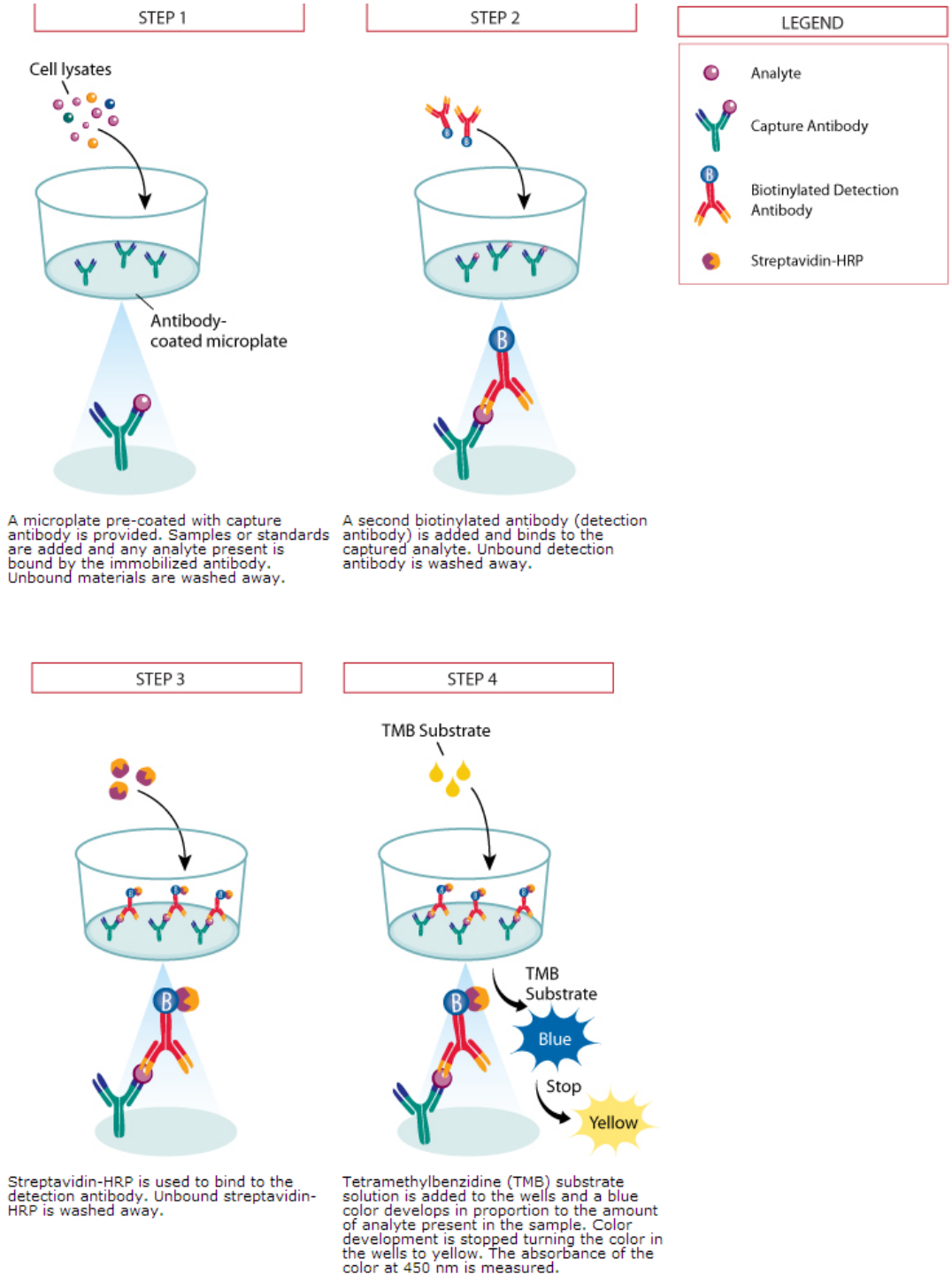



**Figure 43: Scheme of how the DuoSet™ ELISA works.** Figure adapted from [384].

Samples tested were serum-free media that had been conditioned by the cells for a 6-hour time period. A longer time point could not be studied because the HUVECs (obtained from Dr. Tosato's lab at the NIH) detached over longer time periods in serum-free conditions. Capture antibody was diluted and 100 µL added to each well to be used. The plate was then covered and left at room temperature overnight. The excess capture antibody was discarded and each well washed 3 times with PBST (0.05% Tween in PBS). 300 µL of prepared BSA solution (diluted DY995) was then added to each well, covered, and left at room temperature for one hour. After washing 3 times with PBST, samples (fresh samples, freshly thawed samples, or freshly prepared standards) were added (100 µL each), covered, and left 2 hours at room temperature. After washing 3 times with PBST, freshly prepared detection antibody, 100 µL/well was added, and left 2 hours at room temperature. After washing 3 times with PBST, freshly prepared streptavidin-HRP solution was added, 100 µL/well, and incubated in the dark at room temperature for 20 minutes. The plate was then washed 3 times with PBST and 100 µL freshly mixed stabilized hydrogen peroxide and stabilized tetramethylbenzidine at a 1: 1 ratio (DY999) was added to each well. Again, the solution was incubated in the dark for 20 minutes at room temperature. Without washing, 50 µL 2N sulfuric acid (DY994) was added to each well and the plate was gently tapped to ensure thorough mixing. The optical density of each well was determined immediately, using a microplate reader set to 450nm.

## Results

In order to increase angiogenesis, a number of groups have studied the effect of the co-culture of bone marrow stromal cells and endothelial cells as discussed in the Introduction [49, 224-226, 320]. In order to study this effect in our FVB-N/J syngeneic transplant system, we first had to develop a procedure for EC isolation from the mice. There are very few published mEC isolation methods [385-387] from a variety of murine

tissues. Initial attempts using these methods or variations of these methods involved plating sorted cells in tissue culture in order to expand them for FACS verification. Sufficient numbers of cells were never achieved as it became evident that the selected cells were largely not adherent to tissue culture plates, even when coated with a variety of different biomolecules. That the desired cell population wasn't adherent was confirmed through direct FACS after MACS, which showed that the primary antibody and magnetic bead combination and ratio was indeed specifically selecting for the CD31 positive cells. The FACS analysis for CD31 positive selection from lungs, kidneys, and bone marrow, sorted both by size and CD31 content (fluorescence) is presented here in both histograph and bar graph form in Figures 44, 45, and 46 respectively.

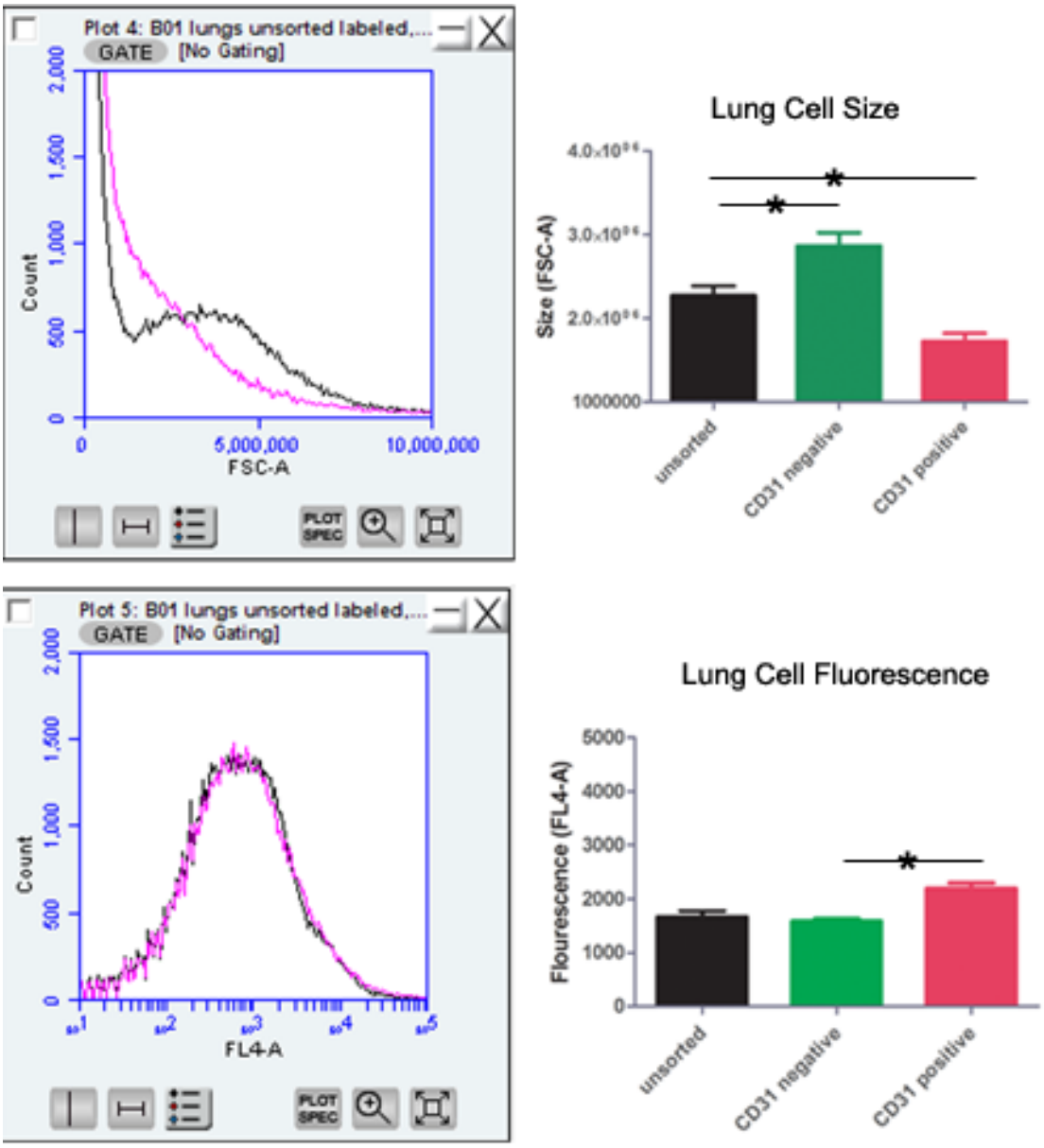



**Figure 44: FACS comparison** by size (FSC-A) and fluorescence (FL4-A) of digested lung cells either unsorted or sorted by MACS for CD31 positive and MACS run-through for CD31 negative cells. * = $p < 0.05$ n = 3 replicates of each MACS condition was tested via FACS

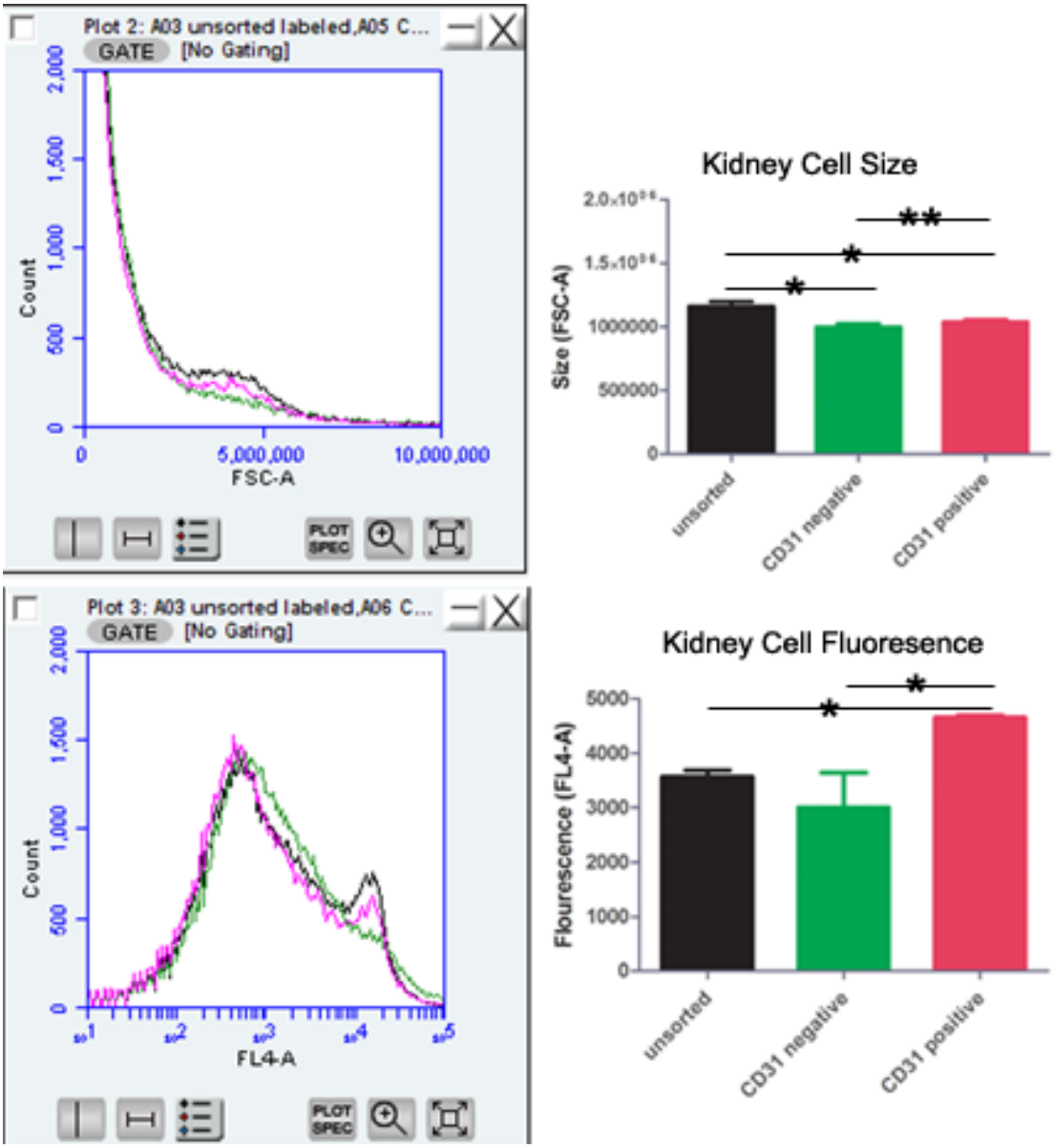



**Figure 45: FACS comparison** by size (FSC-A) and fluorescence (FL4-A) of digested kidney cells either unsorted or sorted by MACS for CD31 positive and MACS run-through for CD31 negative cells. * = $p < 0.05$, ** = $p < 0.01$ n = 3 replicates of each MACS condition was tested via FACS

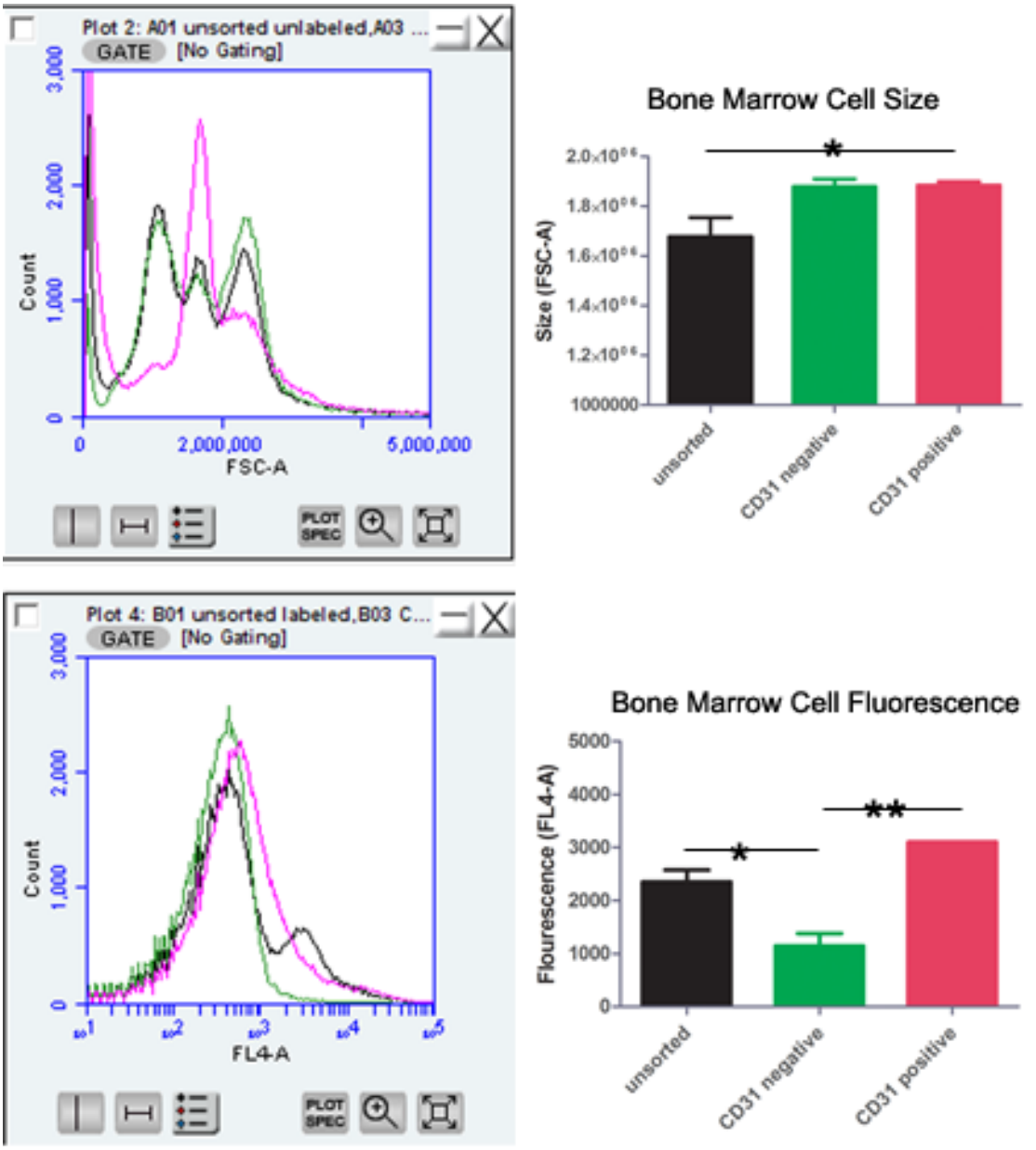



**Figure 46: FACS comparison** by size (FSC-A) and fluorescence (FL4-A) of flushed bone marrow cells either unsorted or sorted by MACS for CD31 positive and MACS run-through for CD31 negative cells. * = $p < 0.05$, ** = $p < 0.01$ n = 3 replicates of each MACS condition was tested via FACS

FACS analysis was performed on the unsorted cells as well as CD31 negative and positive populations. During the MACS analysis, the positive cells were magnetically labeled; thus, the CD31 negative population consisted of the run-through and the CD31 positive population consisted of the final expulsion of cells after removal of the MACS column from the magnet.

Although we demonstrated selection for a discrete population of cells from all tissues both by size differences and fluorescence, the isolation of endothelial cells from bone marrow was the most efficient. As shown in the graph of cell size, the CD31 positive population presents a spike in cells around $1.9x10^6$ on the FSC-A scale; whereas, the CD31 negative population is reduced in cells of this size and so, relatively increases the numbers of cells around $1.2\ x10^6$ and $2.2\ x10^6$ on the FSC-A scale. Therefore, our size measurements indicate that we are selecting for a specific population of cells, and the fact that this population is indeed the CD31 positive population was confirmed by a significant increase in fluorescent labeling. The fluorescent labeling was from an anti-CD31 APC-conjugated antibody measured in the FL4-A channel. Because the bone marrow-derived ECs were more definitive in the FACS results, as well as the relevancy of using bone marrow-derived ECs together with BMSCs, we chose to use these bone marrow-derived ECs for our *in vivo* transplant experiment.

Problematically, as stated above, it became evident that once sorted, mouse-derived ECs are not adherent, either on tissue-culture treated plastic, fibronectin-treated plastic, gelatin-treated plastic, or collagen I-treated plastic. Therefore, our FVB-N/J *in vivo* transplants for this chapter involved a two-step process. Approximately 2-3 weeks prior to transplant, bone marrow cells were flushed from five mice, pooled, and cultured as described. Then, on the day of transplantation, a dozen mice were euthanized and their bone marrow flushed. This large quantity of bone marrow was then sorted via MACS and FACS verified to obtain the CD31 positive population, which was then seeded into scaffolds along with the trypsinized BMSCs.

For our *in vivo* transplant model, we used FVB-N/J BMSCs that had been isolated and expanded for 2 passages in culture. FVB-N/J ECs were isolated from bone marrow the day of transplantation, sorted via MACS using anti-biotin and streptavidin beads consecutively, and verified by FACS. In this experiment, three different cell conditions

were tested: $2x10^6$ mBMSCs alone, $1x10^6$ mBMSCs mixed with $1x10^6$ mECs, and $2x10^6$ mECs alone. Three different materials were tested: collagen crosslinked by DHT, Gelfoam™ crosslinked by DHT, and Gelfoam™. Constructs were transplanted into FVB-N/J mice (i.e., syngeneically) and harvested after 12 weeks. Results from this experiment were inconclusive because the positive control of $2x10^6$ mBMSC in Gelfoam™, demonstrated as a good bone former in the previous experiment, did not form bone.

In order to examine the effect of the co-culture of BMSCs and ECs of human derivation on transplant angiogenesis and bone formation *in vivo*, a similar experiment was performed using hBMSCs obtained from the NIH Bone Marrow Stromal Cell Transplantation Center (Dr. Marianna Sabatino) and human umbilical vein endothelial cells (HUVECs) purchased from Lonza. All cells were seeded in unmodified Gelfoam™. Three different cell conditions were tested: $2x10^6$ hBMSCs alone, $1x10^6$ hBMSCs mixed with $1x10^6$ HUVECs, and $2x10^6$ HUVECs alone. Constructs were transplanted subcutaneously in the backs of NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ mice from Jackson Laboratories (selected to replace the NIH III nude mice used previously) and harvested after 8 weeks. Like the human constructs described in the previous experiment in NIH III nude mice, hBMSCs transplanted in Gelfoam™ into immunocompromised mice failed to form bone, though they did form fibrous deposits and possibly pre-osteoid tissue. Co-culture with HUVECs did not appear to improve the resultant morphology.

As mentioned, many investigators have tried to study the effects of EC and BMSC co-culture [49, 224-226, 320]. However, the field has not fully elucidated whether the interaction is due to secreted growth factors or direct cell to cell contact as discussed in the Introduction [227, 320]. In order to study the effect of co-culture (cell-cell contact) or conditioned media (secreted factors), a standard osteogenic assay was performed. Three cell conditions were tested: hBMSCs, HUVECs, or an initial seeding of equal

numbers of the two cell types. Four basic types of media were tested: BMSC medium, EC-conditioned BMSC medium, EC medium, and EC-conditioned EC medium. Additionally, all combinations of cells with media were tested with and without the addition of osteogenic factors (β-GP, AAP, and Dex). 14 days after addition of osteogenic factors, the experiment was stopped and samples were fixed and stained with alizarin red. Shown here are the alizarin red S scans and 2x magnification images (Figures 47 and 48) as well as quantification of the staining (Figure 49), indicative of calcification.

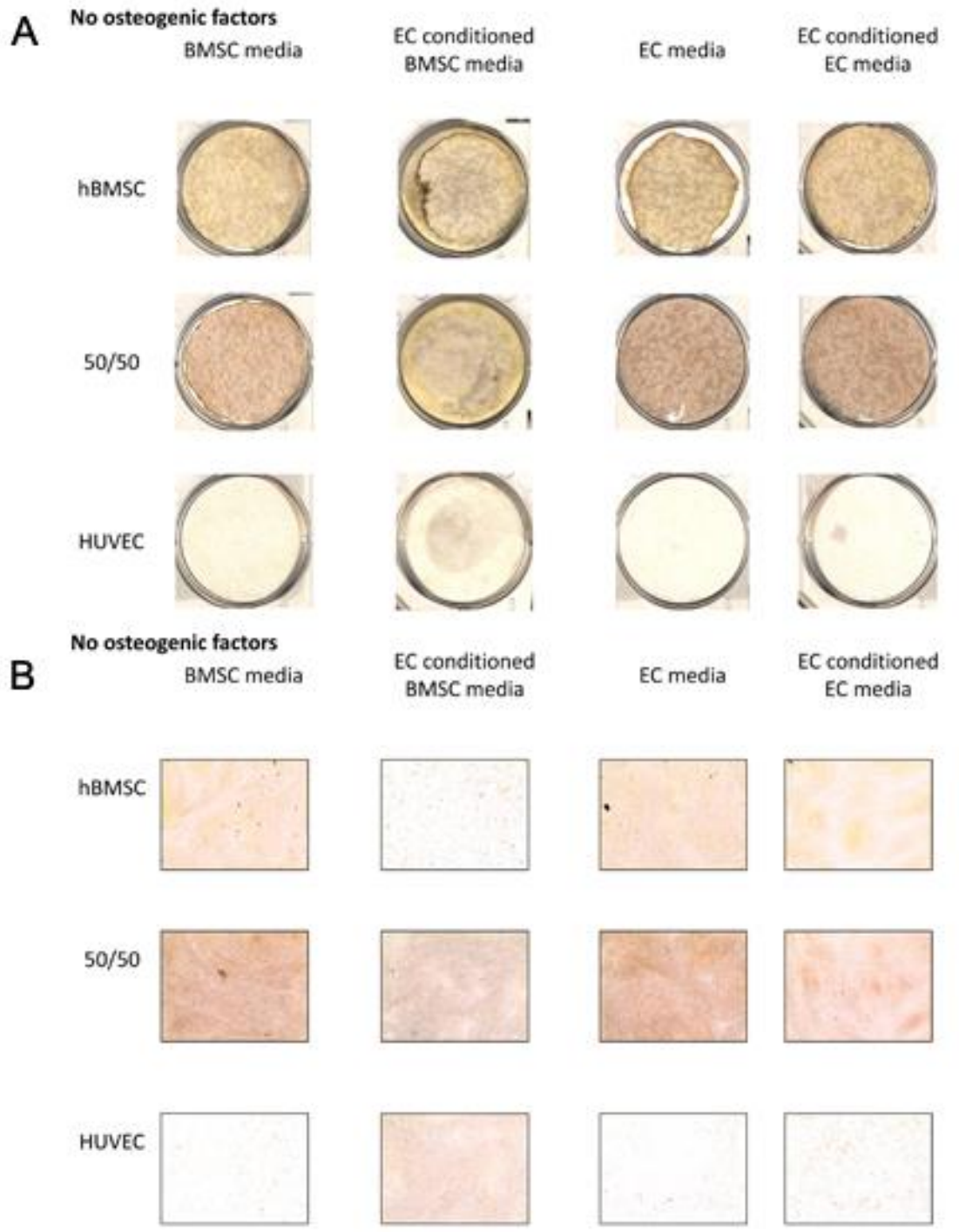



**Figure 47: A) Scans and B) 2x magnification images of samples stained with alizarin red S cultured in the absence of osteogenic factors.**

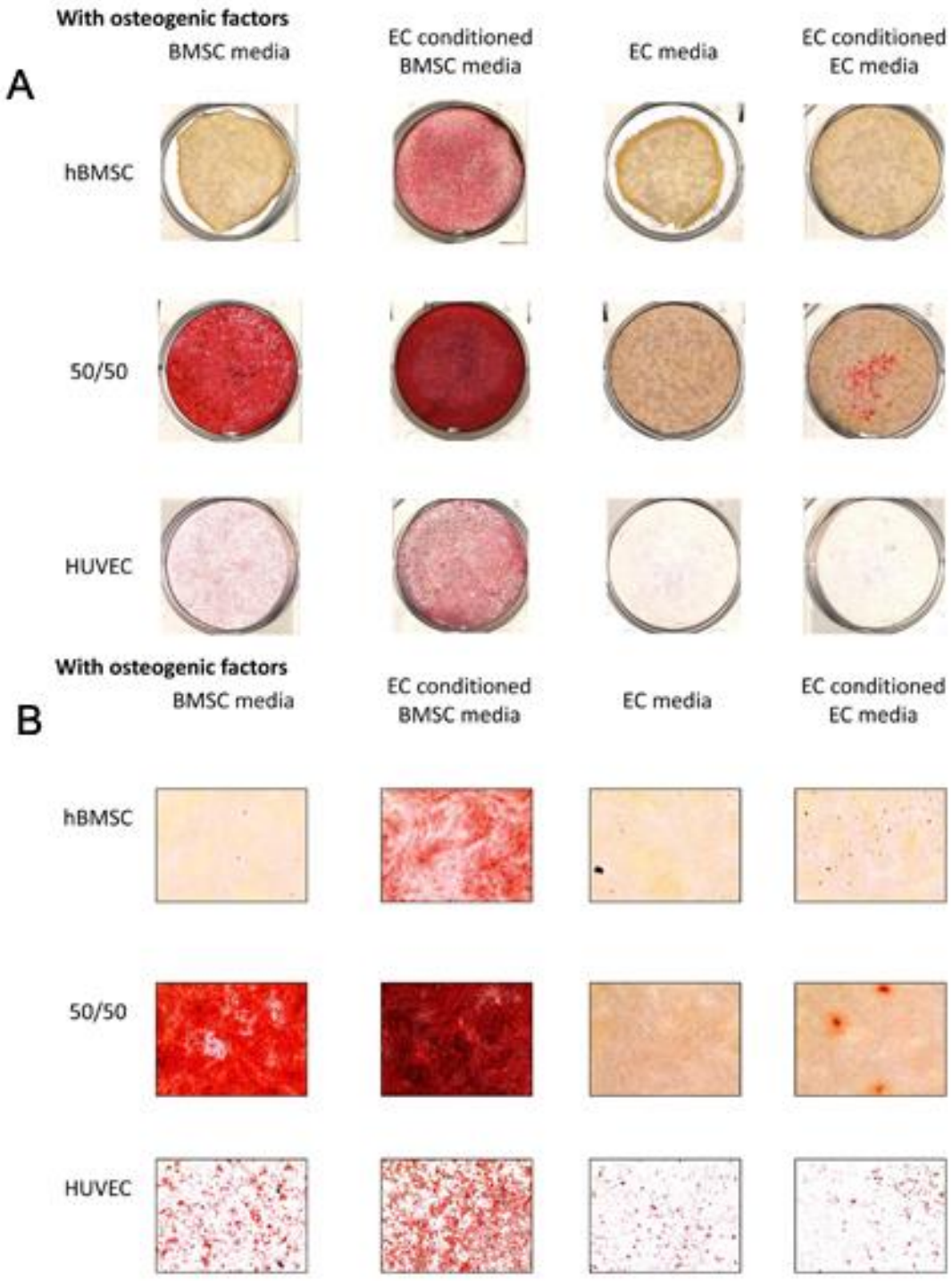
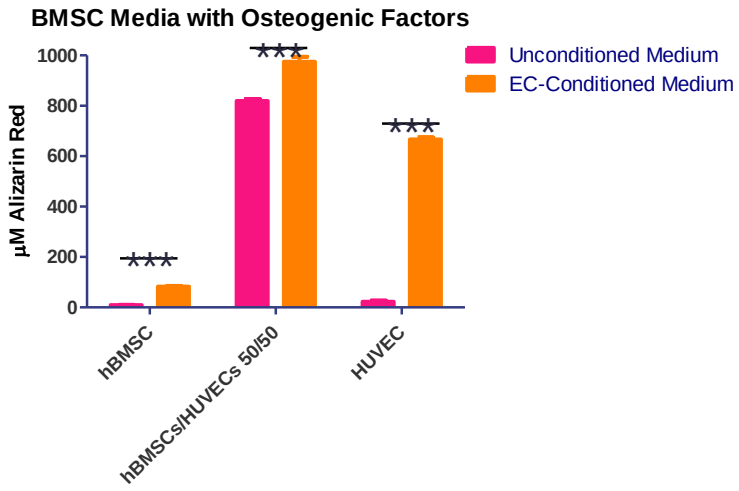



**Figure 48: A) Scans and B) 2x magnification images of samples stained with alizarin red S cultured in the presence of osteogenic factors.**

Figures 47 and 48 demonstrate in images what is quantified in Figure 49. HUVECs and/or hBMSCs grown on gelatin-coated tissue culture plastic do not calcify

when grown in the absence of a cocktail of osteogenic differentiation factors. Interestingly, and in support of our hypothesis that co-culture of cells would enhance osteogenesis, the co-culture of cells calcified several times more than the hBMSCs by themselves. Additionally, growing the cells in BMSC media improved calcification over EC media. Conditioning the media with ECs prior to application in the assay also increased calcification.

**A**




**B**

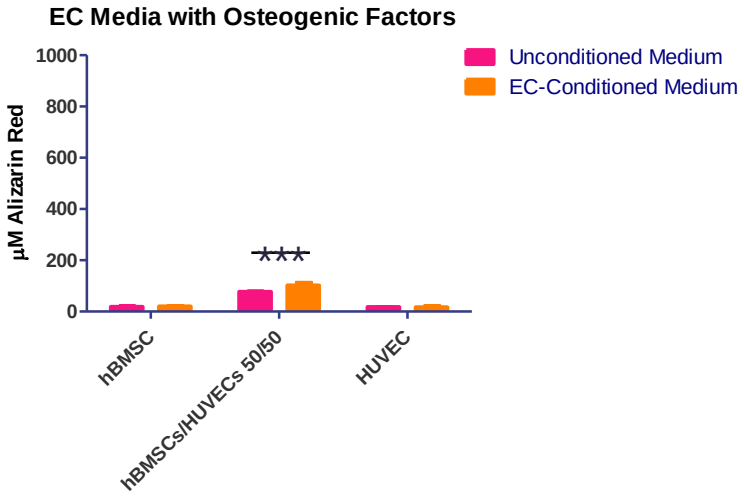



**Figure 49: Quantification of the alizarin red S staining** of samples grown with BMSCs media (A) or EC media (B). *** = $p < 0.001$ n = 3 wells of each condition were used for alizarin red staining

Following the initial experiment, four conditions with osteogenic factors (β-GP, AAP, and Dex) were picked for further analysis: hBMSCs in hBMSC medium, hBMSCs in EC-conditioned hBMSC medium, co-culture in hBMSC medium, and co-culture in EC-conditioned hBMSC medium. Six replicates of each of these conditions were set up. Upon completion (day 10), 3 replicates were fixed and stained and the other 3 were lysed and RNA (and hence, cDNA) was obtained for qPCR.

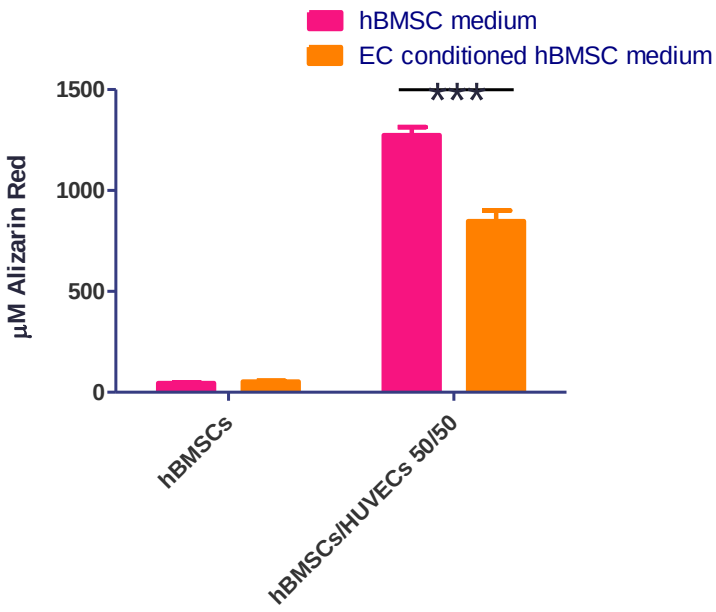



**Figure 50: Quantification of alizarin red staining** from the follow-up osteogenic assay. Difference between hBMSCs and mixed cells is also significant ($p < 0.05$). *** = $p < 0.001$ n = 3 wells of each condition were used for alizarin red staining

The alizarin red S results (Figure 50) show around a 10-fold increase in calcification in the co-culture as contrasted to hBMSCs by themselves. The same result was obtained in our previous experiment shown in Figure 49. However, in this experiment the conditioned medium led to a marked decrease in calcification in the co-culture. qPCR analysis was carried out for osteogenic factors: Runx2, alkaline phosphatase, bone sialoprotein, osteocalcin, and osterix.

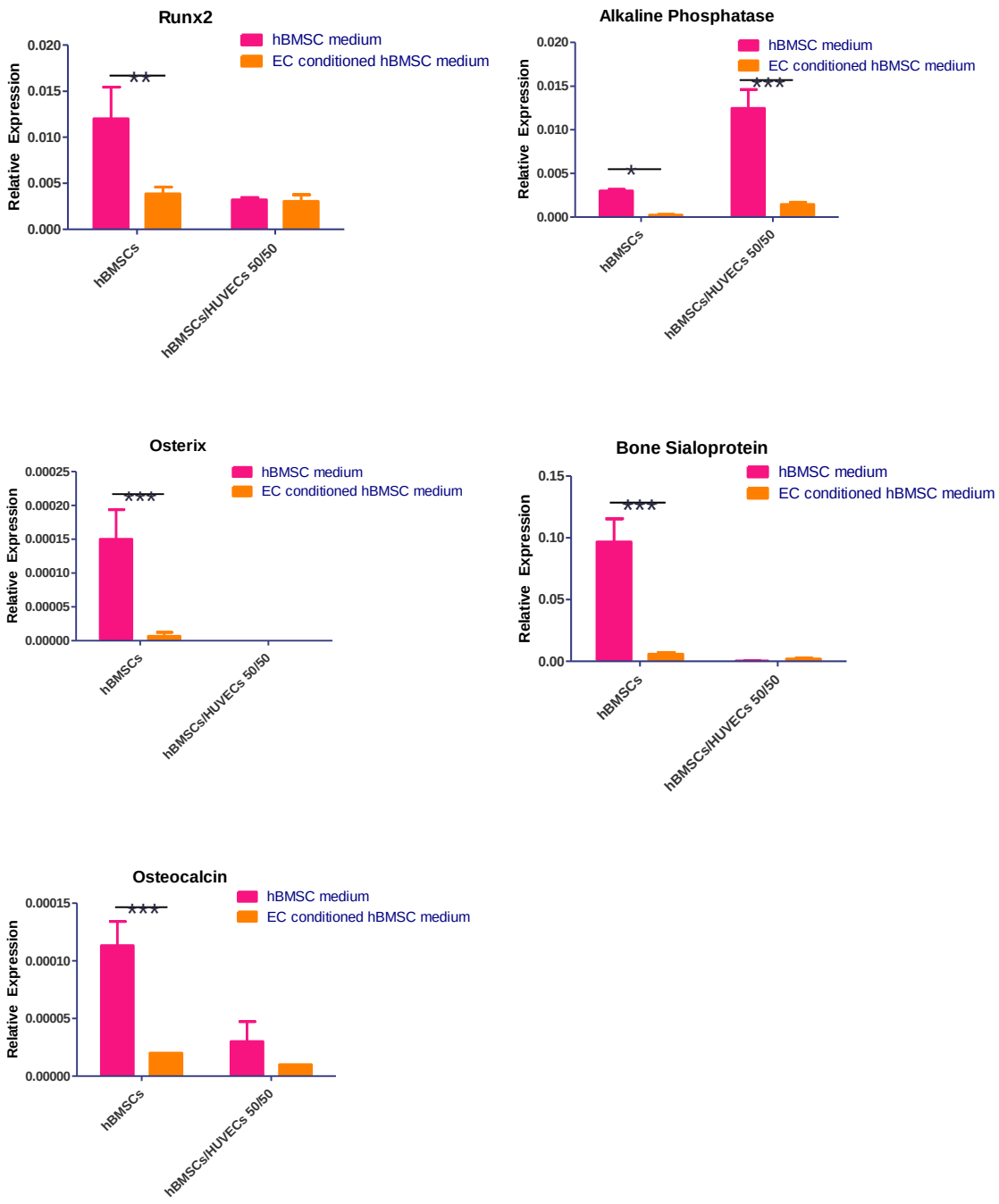



**Figure 51: qPCR results for osteogenic markers** from the follow-up osteogenic assay – performed in triplicate. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ n = 3 wells of each condition were used for qPCR analysis, and each well was tested via qPCR in triplicate in order to minimize pipetting error

qPCR can give insights into cell commitment instead of simply sheer amount of calcification. All genes tested are linked to osteogenic differentiation, as shown in Figure 15. In almost all cases, EC-conditioned hBMSC medium resulted in lower osteogenic gene expression than fresh hBMSC medium. With the exception of alkaline phosphatase

expression where the co-culture was 5 fold higher than mono-culture, mono-culture had higher levels of osteogenic gene expression.

One way of measuring how co-culture can change cell behavior is to measure a change in growth factors secreted in co-culture as compared to individual cultures. Also, in order to study the effect of added growth factors to the system, it is desirable to first understand the endogenous levels of protein secretion. To satisfy both of these aims, ELISAs were performed to measure the secretion of VEGF and TGF-β from hBMSCs and HUVECs over 18 hours, and also whether production of these factors changes upon co-culture of the two cell types.

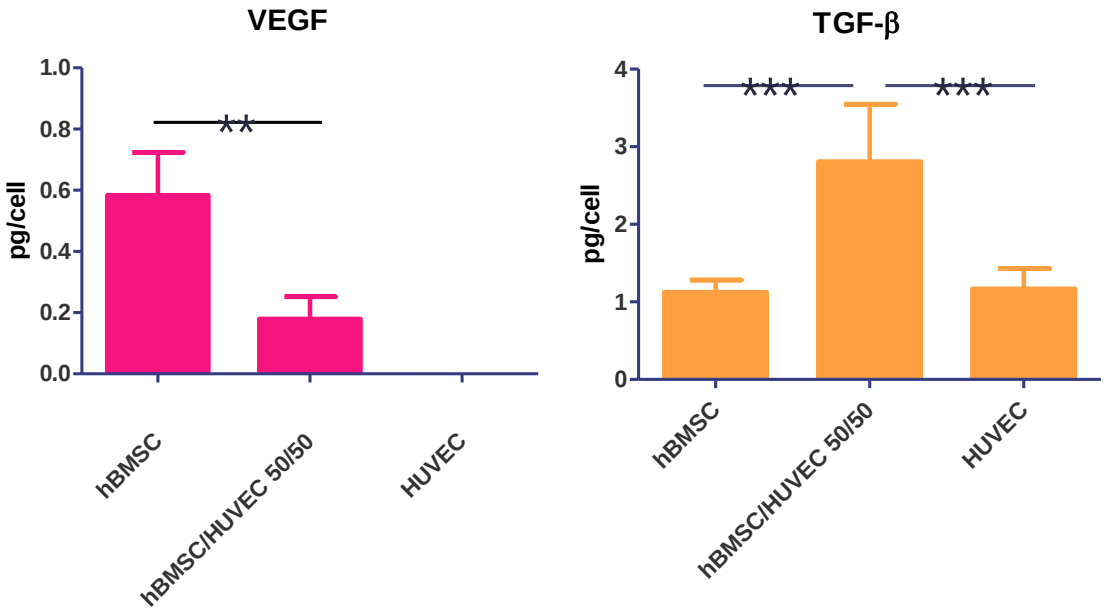



**Figure 52: Results from VEGF and TGF-β1 ELISA assays** measuring growth factor secretion from hBMSCs, HUVECs, and a co-culture over a total of 18 hours. Note the difference in Y-axis scale. ** = $p < 0.01$, *** = $p < 0.001$ n = 3 wells of each condition were used for the ELISA, and each well was tested via ELISA in triplicate in order to minimize pipetting error

Figure 52 shows the ELISA results measuring basal expression of VEGF and TGF-β in hBMSCs, HUVECs, and co-culture. VEGF secretion is approximately 50% lower in the co-culture, and as expected, HUVECs did not secrete VEGF. In contrast, co-culture

expression of TGF-β is significantly higher than either cell type alone, while hBMSCs and HUVECs in mono-culture secrete roughly the same amount.

## Discussion

If we were to use our syngeneic mouse model for the testing of the combination of BMSCs and ECs a protocol for the isolation of FVB-N/J-derived mouse endothelial cells (mECs) needed to be devised. Not only are mECs commercially unavailable (unlike human ECs), there are few published methods for isolation of mECs. Despite initial setbacks, mECs were isolated (via MACS) and verified (via FACS) from mouse lungs, kidneys, and bone marrow. ECs from all three sources were analyzed both in terms of size and fluorescence. While lungs and kidneys (Figures 44 and 45) could both be enriched for CD31 positive populations, the difference in the enriched populations for size and CD31 expression was most efficient in sorted bone marrow (Figure 46). Bone marrow-derived CD31 positive cells were the EC of choice for the main experiments because they would be used in conjunction with BMSCs. ECs from bone marrow would be conditioned to interact with BMSCs; whereas ECs from lungs, for example, might possess memory of interaction with lung epithelial cells and therefore behave differently. Much work has been done has been done on cell memory, particularly in induced pluripotent stem cells [388], but also with regards to CFU-Fs [193] and it was preferable to use bone marrow-derived ECs if possible (which was demonstrably so).

Unfortunately, although this experiment was grand in scale and elegant in design, it did not answer the question of whether or not adding ECs to BMSCs improves performance of the transplant system. The results were inconclusive because the positive control of FVB-N/J BMSCs on Gelfoam™ alone, while very successful in the previous experiment, failed to form any bone or support hematopoiesis in this instance (as did almost all of the transplants). While disappointing, the lack of results in this experiment reflect the inherent difficulties in working with mouse BMSCs, which are

notorious for their finicky nature from one preparation to another. In this instance of seemingly ineffective BMSCs, the ECs did not seem to improve performance, but it can also not be said that they decreased performance of the scaffold, since the scaffold did not perform at all. Thus, it cannot be speculated about whether, given osteogenic BMSCs, the addition of ECs would boost angiogenesis *in vivo*, decrease bone formation, or have any effect at all.

A similar result was obtained for the study of the effect of BMSC and EC co-culture using human cells. This result was less unexpected than for the syngeneic co-culture experiment, because of the results in Chapter 4. In Figures 37, 38, and 39, we demonstrated that hBMSCs on Gelfoam™ in NIH III nude mice failed to form bone. Although the strain of immunocompromised mouse was different in this experiment, the hBMSCs and Gelfoam™ material remained the same. It was hypothesized that the addition of HUVECs to the seeded cell population would increase angiogenesis and therefore stimulate bone formation where none was expected, but this did not occur.

As mentioned above, many researchers in the past have studied the effects of co-culture on osteogenic potential [87]. We used a standard *in vitro* osteogenic assay to study whether direct contact of the cells or conditioned media would have an effect on osteogenic potential when an industry-established cocktail of factors was added [389]. This experiment is a direct response to the results published by Meury *et al*. [389], who also studied the effect of co-culture on *in vitro* calcification.

Alizarin red S staining of our experiment indicates that the osteogenic differentiation is limited in the absence of added osteogenic factors (β-GP, AAP, and Dex) across all samples – a result supported by Meury *et al*. who found that ECs did not induce BMSCs to calcify in the absence of osteogenic medium. We tested both EC and BMSC media with all cell populations in order to maintain respective cell viabilities. However, even with osteogenic factors cells in EC medium, cultures did not calcify

nearly as much as in BMSC medium. Lack of calcification in EC media-treated wells could be either due to the stimulating effect of the different serum used in the BMSC media, or due to inhibitory effects from the ECGS or various serums used in the EC media. ECs in general did not fare well in the assay as demonstrated by their sparse nature observed in the scans and images of the stained samples. EC underperformance was due in part to their density-dependent nature as the assay stipulates that cells are plated then changed to osteogenic culture conditions once the cells are confluent, whereas HUVECs, by their nature, change gene expression and start to undergo apoptosis when over confluent (empirical observation). Additionally, media in the osteogenic assay was only changed every 4 days as contrasted to normal HUVEC conditions of 2 days. However, HUVECs cultured with osteogenic factors did indeed calcify after 14 days when in BMSC media with osteogenic supplements. The co-culture of cell types demonstrated over five times more calcification than the BMSCs by themselves, showing that direct co-culture stimulates, rather than hinders calcification *in vitro*. Our use of conditioned media to study the indirect effect of secreted growth factors from endothelial cells resulted in an increase in calcification in cells with conditioned medium over unconditioned medium.

Because the initial experiment had tested so many different conditions, a smaller study was conducted to verify that endothelial cells increase the osteogenic potential of transplants both through direct and indirect action. Four conditions were therefore tested, all under the influence of osteogenic factors: BMSCs in BMSC medium (control), BMSCs in EC-conditioned BMSC medium (to test for the effect of secreted growth factors), co-culture of BMSCs and HUVECs in BMSC medium (to test for the effect of direct cell contact), and co-culture of the cells in HUVEC-conditioned BMSC medium (combined direct and indirect contact). These conditions were repeated in six wells, three of which were used for alizarin red S staining, and three for qPCR.

The alizarin red S results from this experiment confirm that a mixed population of endothelial cells and bone marrow stromal cells cultured together has significant, up to 10-fold in the case of un-conditioned media, increase in the ability to calcify as contrasted to BMSCs grown alone. This result has since been confirmed by Thebaud *et al*. who ran a similar assay for 21 days and found increased von Kossa staining (indicative of calcium deposits) in the co-culture as contrasted to either cell type individually [390]. Interestingly, our results indicate that EC-conditioned BMSC medium had a negative effect on the calcification potential of the co-culture, although this effect was not observed with BMSCs grown alone in this medium. In this instance, we can hypothesize that as a net effect, conditioning the medium removed more calcification stimulus from the solution than was added by the conditioning cells.

qPCR analysis of osteogenic markers in these conditions was performed and several trends were noted. BMSCs grown with BMSC media and osteogenic supplements always had higher expression of these markers than when grown with conditioned media. There are two possible explanations for this observation. The first explanation is that conditioned media is, to a certain extent, "spent media." The media had previously been placed above ECs in culture for 3 days in order to condition it. While the ECs during that time had secreted growth factors into the media in order to enrich the media in a unique way, they had also depleted the serum and other molecules present in the media as made, in order to sustain their continued growth. Because it is impossible to know the amount of serum and other factors depleted it is therefore impossible to supplement the media to correct for this in a non-arbitrary fashion (and hence such correction was not attempted). The second explanation with regards to the factors and signals secreted by ECs during the conditioning process is as follows. HUVECs, understandably, prefer to grow in their standard EC media as contrasted to BMSC media. Therefore, one might reasonably argue that the factors secreted by conditioning cells into the media differed between the two types of media. Likewise,

although this was not shown in our first experiment, conditioned media appears to have a negative effect on osteogenic potential as demonstrated both by alizarin red S staining for actual calcification of the sample, as well as a decrease in expression of all osteogenic markers tested.

Analysis of our results from un-conditioned medium treated samples shows that for runx2, osteocalcin, osterix, and bone sialoprotein (BSP), expression by hBMSCs was higher than expression in the co-culture. The only osteogenic marker tested where the co-culture expressed higher levels than BMSCs alone, was alkaline phosphatase. This finding is in direct contradiction to the results of Meury *et al*. [389] who found a decrease in ALP (amongst other markers) when HUVECs were in direct contact with the hBMSCs. However, Meury *et al*.'s result in this instance appears to be against the prevailing literature. Our results confirm the finding by Villars *et al*. who found that direct co-culture increased alkaline phosphatase activity in co-culture [391], and contended that this increase would only occur when direct gap junctions between the two cell types were present. Saleh *et al*. showed that this increase in alkaline phosphatase occurred in their 3D *in vitro* model [392]. Upon further analysis, however, this result obtained from the qPCR is counter-intuitive to the result that was seen by alizarin red S staining. Alkaline phosphatase is an early marker of bone formation. In contrast, BSP is a mid-stage marker, and osteocalcin is a late stage marker [262] (general gene expression patters are shown in Figure 15). Consequently, the results of the calcification assay do not match the levels of expression of the osteogenic markers. One would have expected to see high levels of the BSP and osteocalcin in the cultures that were heavily calcified (co-cultures) if this calcification were actually a true indicator of osteogenic differentiation; however, such expression was not observed. The explanation for these results most likely derives from the finding that alkaline phosphatase was dramatically increased in BMSC/HUVEC co-cultures. Alkaline phosphatase can cleave β-glycerophosphate, thereby generating free phosphate that can

readily bind calcium in the medium and form a calcium phosphate precipitate. However, alizarin red S cannot distinguish between calcium phosphate precipitates and hydroxyapatite that would be formed in the case of cell-mediated matrix mineralization. Alizarin red S can also stain dystrophic calcification induced by dead and dying cells. Thus, we hypothesize that the calcification of the un-conditioned medium samples presented here represent dystrophic calcification and not true osteogenic differentiation; and propose that alizarin red results in and of themselves should always be placed in the context of supporting experiments to distinguish between these two effects. Another, simpler method that was used to measure the interaction between hBMSCs and HUVECs was ELISA testing of the growth factors secreted from these cells when cultured individually or together. Secreted factors allow cells to communicate locally as well as to recruit other cells to the system. Cells can be stimulated to secrete growth factors either by direct contact with other cell types (and communication through non-secreted pathways), or through uptake of signaling factors from the extracellular space. For this study we analyzed VEGF and TGF-β production was analyzed using ELISA kits from R&D Systems. These two factors were selected since they would be tested in other *in vitro* studies – BMP-4 and heparin were not tested because ELISA kits for these molecules are not available. Unlike Li *et al*.[393], self-assembly of co-cultured cells into defined structures were not observed, either in this study or in the osteogenic assay above, but this could be due to the high density of the cultures, as contrasted to their experiments.

The results from the VEGF ELISA study indicate that VEGF is not secreted by ECs. This result was expected as studies have shown that ECs do not themselves produce VEGF, but rather respond to VEGF secreted by surrounding tissue [290-292]. The result from the co-culture is an additive response, in that expression is simply a sum of expression from each cell type. Remarkably, our results show approximately 0.8 pg of VEGF secreted per hBMSC over a period of six hours, working out to 3.2 μg per million

cells per day, significantly more than Kaigler *et al*.'s findings of 3.2 ng per million cells per day [87]. This difference could be explained by the fact that the calculations presented here were measured over 6 hours, and then extrapolated (assumption of steady VEGF secretion might be incorrect), whereas Kaigler *et al.* measured over 16 hours and then extrapolated. Additionally, Kaigler *et al.* maintained their culture in "basal medium" but admit that this included 1% FBS, enough to drastically change the behavior of cells. In this context, it could be hypothesized that the higher level of VEGF secretion in this experiment might partly be a response to the total lack of FBS in the culture conditions.

The results from the TGF-β ELISA indicate that, while hBMSCs and HUVECs do not significantly differ in their TGF-β production, the co-culture produces approximately three times as much TGF-β. The higher reading indicates that the combination of cells is synergistic, not additive, and that some sort of interaction is occurring to increase production. Either hBMSCs are stimulating HUVECs to produce more TGF-β, or vice versa, or each of them is stimulating the other. An equivalent to this finding cannot be found in the literature, primarily because most studies of HUVEC and hBMSC co-culture focus on VEGF mediated behaviors. TGF-β stimulates proliferation of endothelial cells and also has been shown to change extracellular matrix production of HUVECs. Thus, we can hypothesize that the observed syngeneic increase in TGF-β secretion might have a positive effect on our tissue-engineered system.

# Chapter 6: How can EC Migration into the Transplant be Increased?

## Introduction

As discussed in the Introduction, it would be desirable to increase both angiogenesis and vasculogenesis of the transplant system. The goal was to establish a vasculature throughout the scaffold as quickly as possible. Therefore, methods to increase both *de novo* formation of blood vessels in the scaffold (i.e., vasculogenesis – the reasoning behind Chapter 5) and growth of blood vessels into the transplant from the host area surrounding the transplant (i.e., angiogenesis – the basis of this chapter) were investigated. The fundamental reasoning is that the first step of angiogenesis is the migration of endogenous endothelial cells towards and into the scaffold. In this chapter angiogenesis models via two *in vitro* studies, the Boyden chamber assay and the 2D motility assay, and via one *in vivo* system, the Directed *In Vivo* Angiogenesis Assay (DIVAA), were utilized. The hypothesis to be tested was that EC movement could be stimulated by selected individual or combined growth factors.

## Methods

### Boyden Chamber Assay

A Boyden chamber assay was run to determine the response of EC migration in response to growth factors secreted from either BMSCs or HUVECs, either indirectly through conditioned medium, or though non-direct contact cell communication with cells on the bottom of the membrane. The basic purpose of a Boyden Chamber assay is to measure the movement of seeded cells across a membrane in response to a stimulus on the opposite side of the membrane. The negative control was basal medium with no supplements on both sides of the membrane. The positive control was of basal medium on the top of the membrane with the seeded cells, and full, normal, FBS and other

growth factor-containing EC medium (described previously) on the bottom of the membrane. In order to control for stimulus migration across the membrane and subsequent equilibration of added stimulus on both sides of the membrane, leading to increased non-directional motility of cells, a control of 74% strength EC medium on both sides of the membrane was also performed (this contains the same amount of stimulus in the total system as the positive control (500μL above the membrane of basal and 1400μL below the membrane of full strength but evenly distributed across the membrane instead of instead of as a gradient). All wells were filled according to manufacturer's instructions in order to prevent osmosis across the membrane due to physical pressure differences. For the experimental samples, basal medium was placed on top of the transwell insert membrane along with HUVECs, whereas either conditioned medium, or pre-attached cells were present in the bottom well and switched to either EC medium or basal medium upon commencement of the experiment. The experiment was run for 24 and 48 hour time points. This experimental setup is shown below:

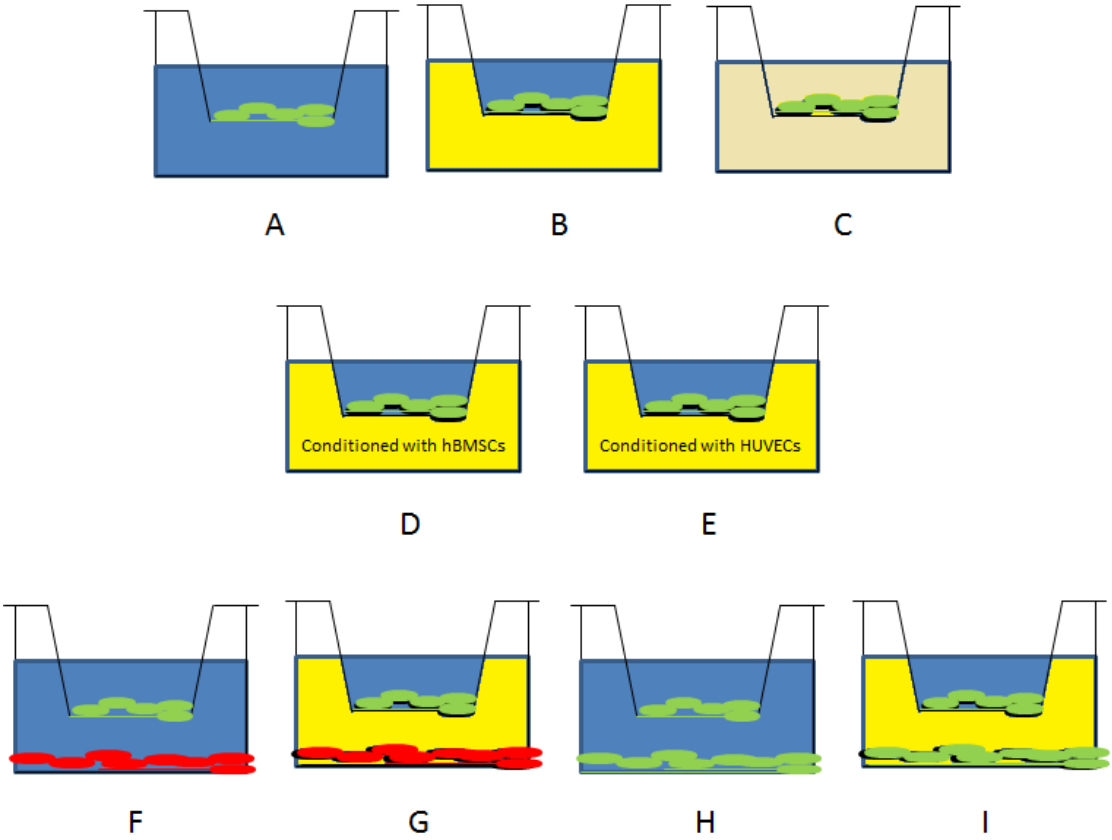



**Figure 53: Setup for Boyden Chamber assay.** Green cells are HUVECs that migrate through the transwell membrane. Red cells are hBMSCs. Blue medium is basal EC medium (M199), and yellow medium is full HUVEC medium. Beige medium is 74% strength EC medium. Thus, from left to right: A) negative control: M199 on both top and bottom, B) positive control: M199 on top, EC medium on bottom, C) random cell motility control: 74% strength EC medium on both top and bottom, D) test sample with basal medium on top, and EC medium pre-conditioned by hBMSCs on the bottom, E) test sample with basal medium on top and EC medium pre-conditioned by HUVECs on the bottom, F) test sample with basal medium on top and bottom, but with additional hBMSCs seeded on the bottom, G) test sample with basal medium on top and full medium on the bottom, but with additional hBMSCs seeded on the bottom, H) test sample with basal medium on top and bottom, but with additional HUVECs seeded on the bottom, I) test sample with basal medium on top and full medium on the bottom, but with additional HUVECs seeded on the bottom

At the end of the experiment, medium on both top and bottom was gently aspirated, and both sides gently washed with PBS three times, followed by incubation in 4% PFA in PBS in order to fix the cells. Samples were then washed three times with PBS to remove any residual fixative, and incubated with 1: 10000 DAPI in TBS with 0.1% Tween for 5 minutes at 37°C. Samples were then washed again three times with PBS and mounted using fluorescence-stabilizing hard-set mounting medium.

Samples were then imaged at 4x magnification, such that 5 images covered most of the area of the membrane with minimal overlap. Images generated were then analyzed with ImageJ. Briefly, data tiles were opened in ImageJ, and brightness and contrast was set to maximum contrast. Images were then converted to binary format and particles under 50 square pixels counted. Results were then exported to Excel and GraphPad for statistical analysis. Examples of the negative and positive controls are shown in fluorescent DAPI image format as well as processed by ImageJ into binary format for quantification in Figure 54.

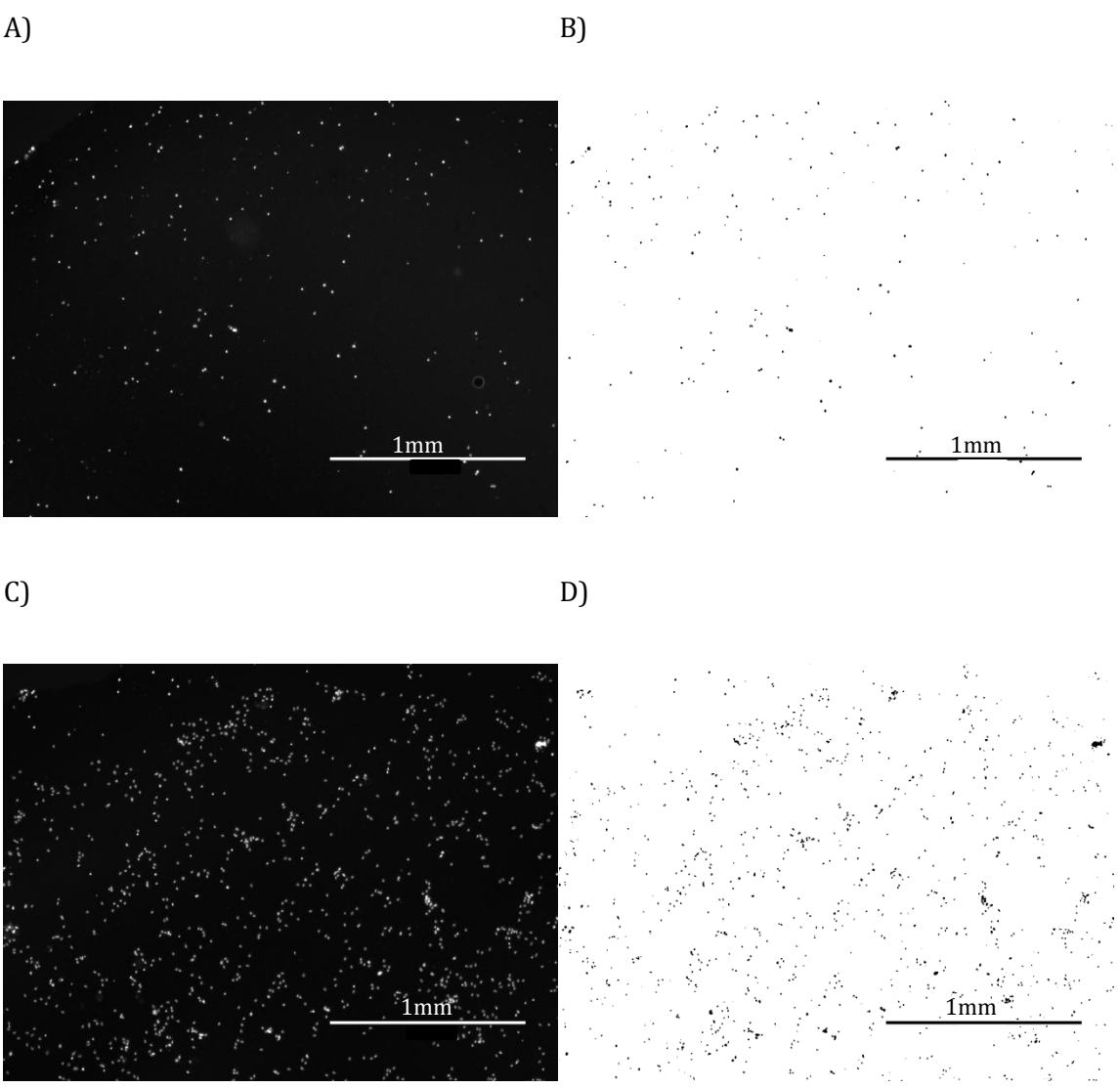



**Figure 54: Images of negative control (A) and positive control (C) in fluorescent DAPI format, and their ImageJ processed counterparts (B and D) for quantification.**

## 2D Motility Assay

### *Experimental Setup*

$7.5x10^4$ cells were plated per 35 mm dish in 2 mL medium, left to adhere and grown overnight, then medium was changed as desired for monitoring. Immediately after changing the medium, dishes were transferred to Zeiss AxioVert incubated time-lapse microscopes and observed over 48 hours through time-lapse photography at 5 minute intervals.

### *Calculating Cell Velocity with ImageJ*

Time-lapse images were opened using the Import Sequence tool in ImageJ at 70% image size and at intervals of every 4 images (translating to 20-minute intervals). The sequence was then converted to 8-bit and cells were tracked using the Manual Tracking plug-in (adjusted to 0.934579 pixels per μm) to obtain raw data. Many cells were individually tracked throughout this period. If cells exited the field of view during the imaged period, the tracking was ended and data up to that time point was used. If a tracked cell divided during this time period, one of the daughter cells was spontaneously randomly chosen and tracking continued. Figure 55 below shows an example of the paths of 2D motility generated from individual cells. The resulting data were then analyzed using the Chemotaxis Tool plug-in from Ibidi for 3-hour intervals. Data were then manually added to an Excel file, formatted, and exported to GraphPad Prism for statistical analysis.

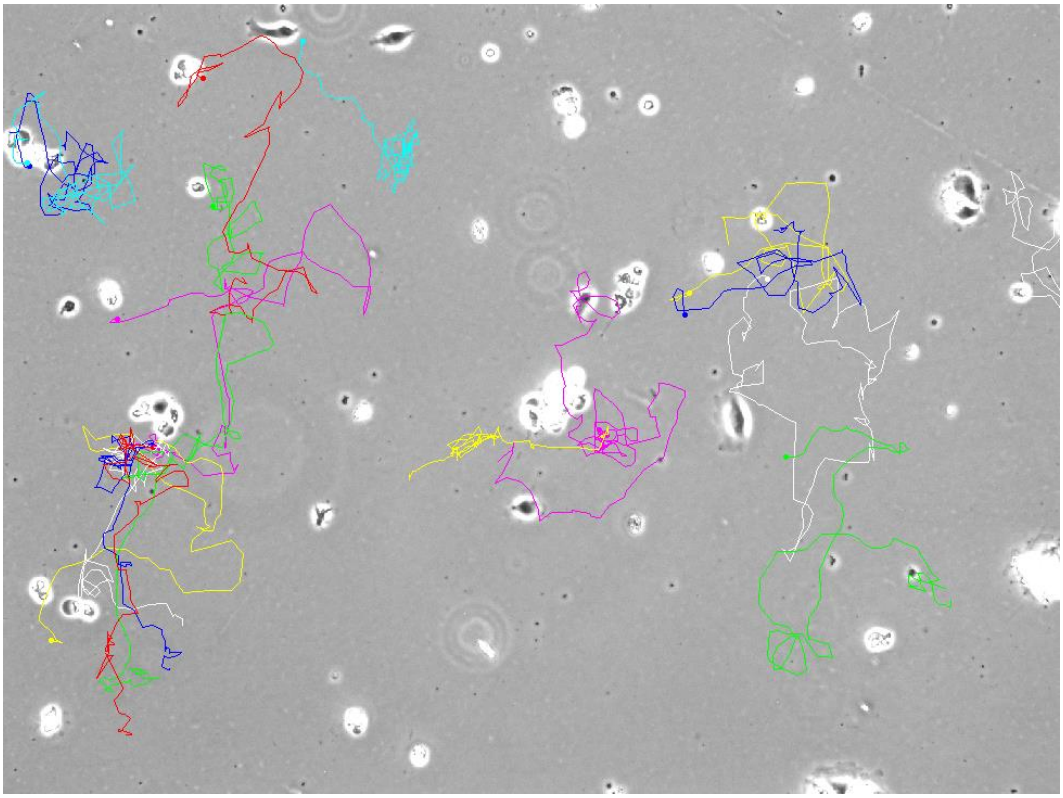


**Figure 55: An example of paths taken by individual cells** over 48 hours in 50 ng/mL VEGF in M199 basal medium.

## Directed In Vivo Angiogenesis Assay

### *Preparation of Transplants*

The Directed *In Vivo* Angiogenesis Assay (DIVAA) was purchased from Trevigen and used according to the manufacturer's instructions. Basically, the assay consists of a tube that is closed at one end. The tube is filled with basement membrane extract (BME) along with any desired growth factors. Blood vessels grow into the tube from the open end in response to the growth factors contained in the tube, and the length of blood vessel penetration can be visually measured. Additionally, post-imaging, the contents of the tube can be dissolved, fluorescent immunohistochemical staining for ECs performed, and quantified via spectrophotometry.

Growth factor-reduced BME was thawed on ice at 4°C overnight. All pipette tips, angioreactors, growth factors, assembly rack etc. were also pre-chilled at 4°C. Working

on ice, angiogenic factors as desired were added (maximum 20 µL) to one tube (200 µL) of Growth Factor-Reduced BME. Each tube of BME is sufficient for 8 angioreactors. Positive control angioreactors consisted of 10 µL of FGF-2 (300ng)/VEGF (100 ng), and 1µl of heparin solution per 200 µL of BME. Negative controls consisted of 11 µL of PBS. Solutions were gently pipetted up and down to mix, without introducing air bubbles. Angioreactors were placed in the AngioRack and 20 µL of BME either containing controls or test samples were added to each angioreactor, using a pre-chilled sterile gel-loading tip, without introduction of air bubbles. Once filled, angioreactors are immediately inverted and transferred to a sterile microtube, then left at 37°C for 1 hour to promote gelation (inverting prevents formation of a meniscus).

### *DIVAA Transplantation Surgery*

DIVAA constructs were transplanted into CrTac: NCr-Foxn1nu homozygous (i.e., nude) mice from Taconic in accordance with NIDCR ASP #10-571. Mice were anaesthetized with isoflurane and administered buprenorphine. Two 1 cm cuts were made using sharp scissors on the dorsal-lateral surface of the mouse back, approximately 1cm above the hip sockets, taking care to leave underlying tissues undisturbed. Blunt scissors are used to create a subcutaneous pocket extending towards the head of the mouse. Just prior to insertion, angioreactors are dipped in PBS to facilitate ease of insertion. Two angioreactors are placed in each pocket, with the open ends furthest from the incision (and closest to the head). All 4 angioreactors in each mouse contain identical growth factors and doses. The incisions were closed with metal clips. Transplants were left in the mice for 14 days.

### *DIVAA Harvesting and Analysis*

Mice were euthanized with $CO_2$ followed by cervical dislocation, and the skin was removed around the transplants. A scalpel was used to sever any vessels growing into the angioreactors. Transplants were then removed and imaged for the purpose of

length of blood vessel penetration measurements. The bottom cap of the angioreactors was removed with a sterile razor blade, and the BME/vessel complex pushed out into a sterile Eppendorf using a 200 µL pipette tip. Each angioreactor was rinsed with 300 µL of CellSperse™ and the fluid was placed with the BME in the microtube. The tube was then capped and incubated at 37°C for 1-3 hours to create a single-cell suspension. The suspension was centrifuged for 5 minutes at 250 x g at room temperature, and the supernatants were discarded. The pellets were resuspended in 500µL DMEM with 10% FBS and incubated at 37°C for 1 hour. The suspension was centrifuged for 10 minutes at 250 x g at room temperature, and the supernatants were discarded. The pellets were resuspended in wash buffer (25 mL DIVAA™ 10X Wash Buffer diluted with deionized water), centrifuged and washed 3 times. The final time, the pellet was resuspended in 200 µL of DIVAA™ FITC-Lectin. This solution is incubated at 4°C overnight. The next day, the solution was centrifuged at 250 x g, and the pellet was washed three times more. The suspension was centrifuged for 10 minutes at 250g at room temperature, and supernatants discarded. The pellets were resuspended in wash buffer (25 mL DIVAA™ 10X Wash Buffer diluted in deionized water), centrifuged and washed 3 times. Finally, the pellets were resuspended in 100 µL of DIVAA™ Wash Buffer and fluorescence was measured at 490 nm.

## Results

### Boyden Chamber Assay

In order to test if HUVECs migrate in response to either secreted factors from HUVECs or hBMSCs (conditioned medium), or via non-contact cell communication to nearby HUVECs or hBMSCs, a Boyden Chamber assay was performed. Migration of HUVECs across the membrane in response to a chemokine gradient was observed.

All conditions demonstrated decreased cell counts at 48 hours compared to 24 hours. The controls demonstrated a response to a gradient of stimulus as demonstrated by the

negative control (basal medium) compared to random control (74% diluted medium) and positive control (full endothelial cell medium) as demonstrated in Figure 56.

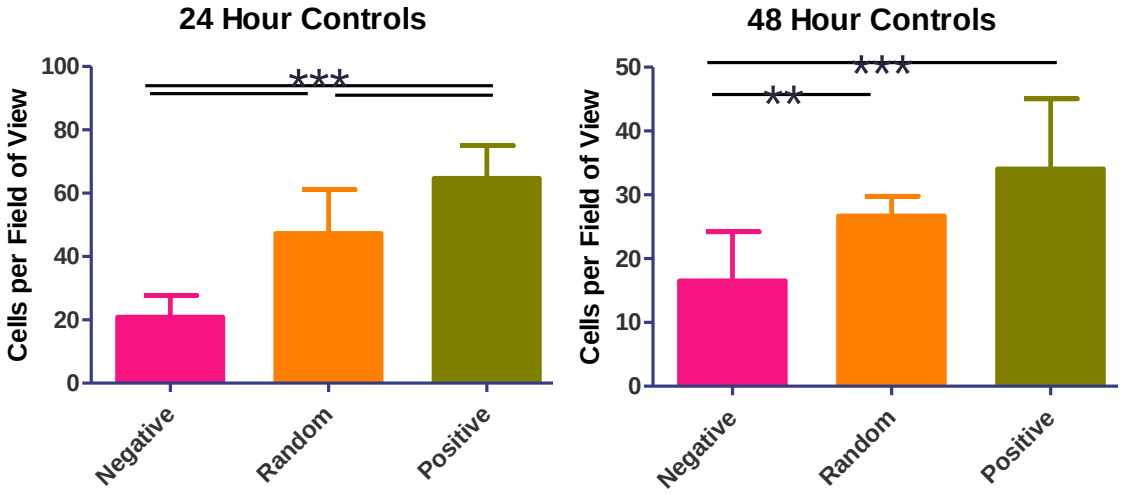



**Figure 56: Migrated cells after 24 and 48 hours.** The negative control was basal medium. The random control was 74% diluted EC medium. The positive control was full EC medium. Note the difference in y-axis scale. ** = $p < 0.01$, *** = $p < 0.001$ n = 6 wells per condition

Figure 57 demonstrates that conditioning the EC medium actually decreased the migration of cells as compared to unconditioned EC medium, though still produced higher migration than basal medium. Conditioning the medium, as discussed in the thesis, not only enriches the medium through secreted growth factors from the conditioning cells, but these same cells also deplete the medium by taking nutrients from it to grow.

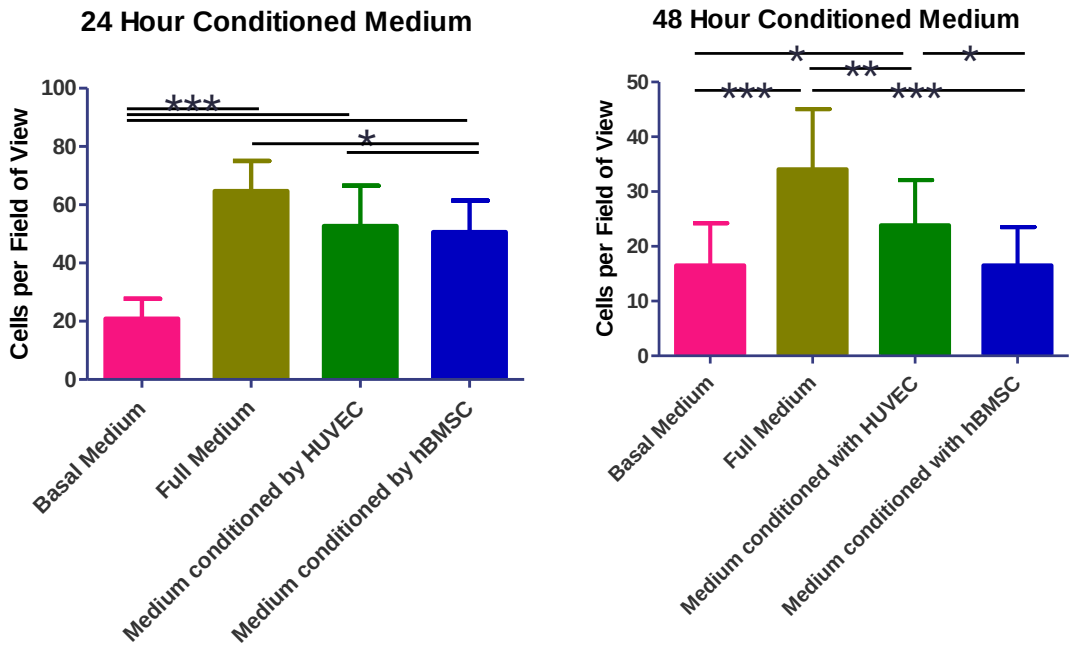



**Figure 57: Effect of conditioning EC medium** with either HUVEC or hBMSC for 2 days. Note the difference in y-axis scale between 24 and 48 hours. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ n = 6 wells per condition

In a more detailed follow-up to the Boyden Chamber Assay presented in the thesis, the effect of culturing cells in the well below the membrane was studied; i.e. to create a non-contact method for the cells to communicate with each other. Although the cells migrating through the membrane were HUVECs, both HUVECs and hBMSCs seeded in the bottom well were tested to measure HUVEC migratory response.

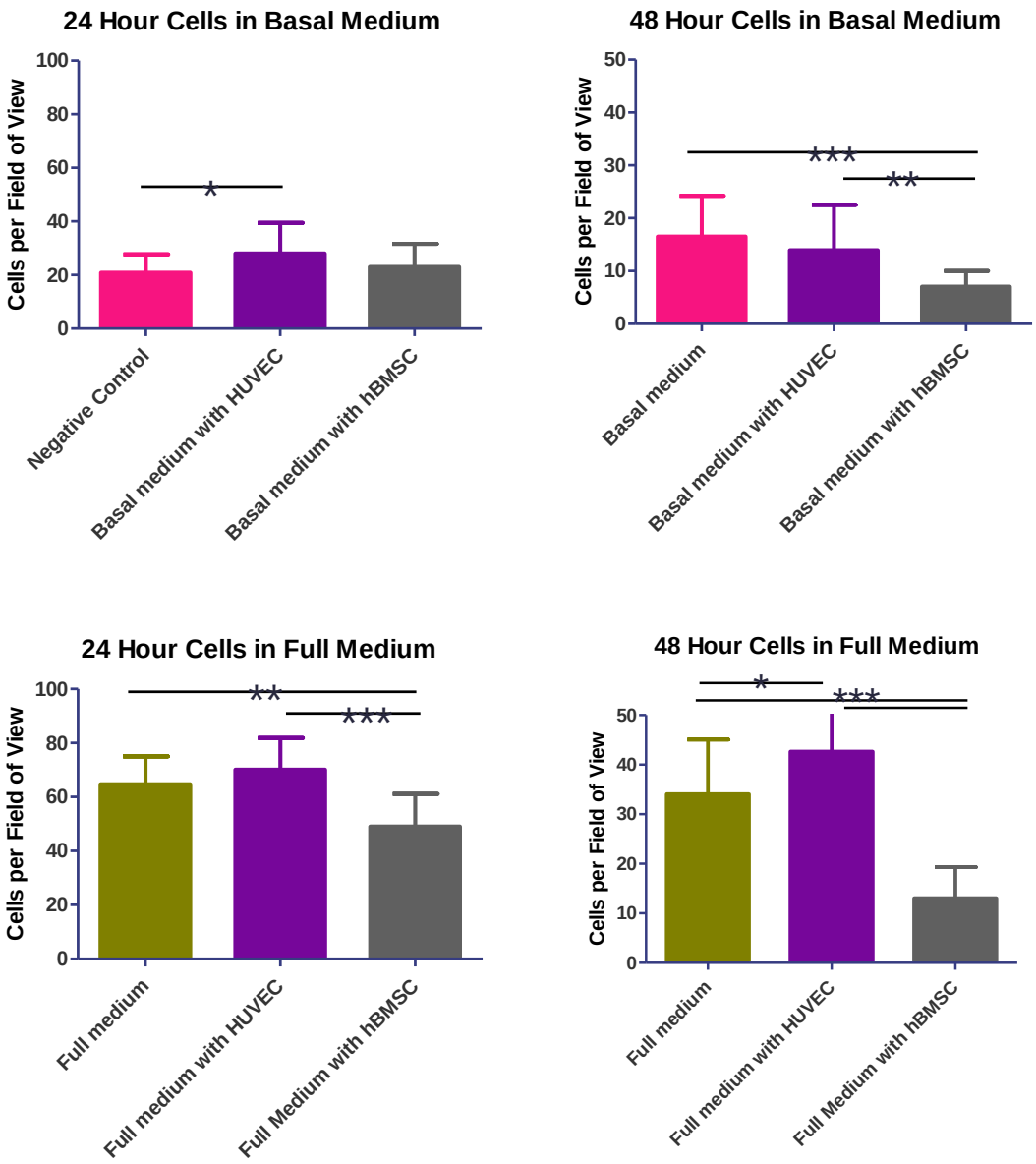



**Figure 58: Effect of non-contact cell communication** on the migration of HUVECs through the membrane. Effect is measured with either basal or full medium underneath the membrane; the cells were always seeded in basal medium. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ n = 6 wells per condition

Migration in response to cells on the bottom was performed with either basal or full medium in the bottom, always with the cells on top seeded in basal medium. The reason for testing both types of medium on the bottom was to increase the viability of the cells for a longer period of time. The results above demonstrate that in basal medium HUVECs did attract HUVECs towards the bottom of the dish by the 24 hour time point, however this effect was no longer present at 48 hours. In full medium, HUVECs in

the bottom well had no effect on HUVEC migration through the membrane by 24 hours; however, an effect appeared at 48 hours. In both cases when full medium was present, and also at 48 hours in basal medium, the presence of hBMSCs in the bottom well decreased HUVEC movement across the membrane compared to the relevant controls.

### 2D Motility Assay

A simple way of measuring the effect of added growth factors at different doses of stimulus is through a 2D motility assay. As described in methods, growth factor of known dosage is added to the media above adherent cells and imaged every 5 minutes over 48 hours in an incubated microscope. A positive control comprised the normal EC growth medium, and the negative control was the basal medium, M199. All growth factors were tested at two different doses in both M199 and EC media as the HUVEC survival after about 36 hours in M199 was minimal.

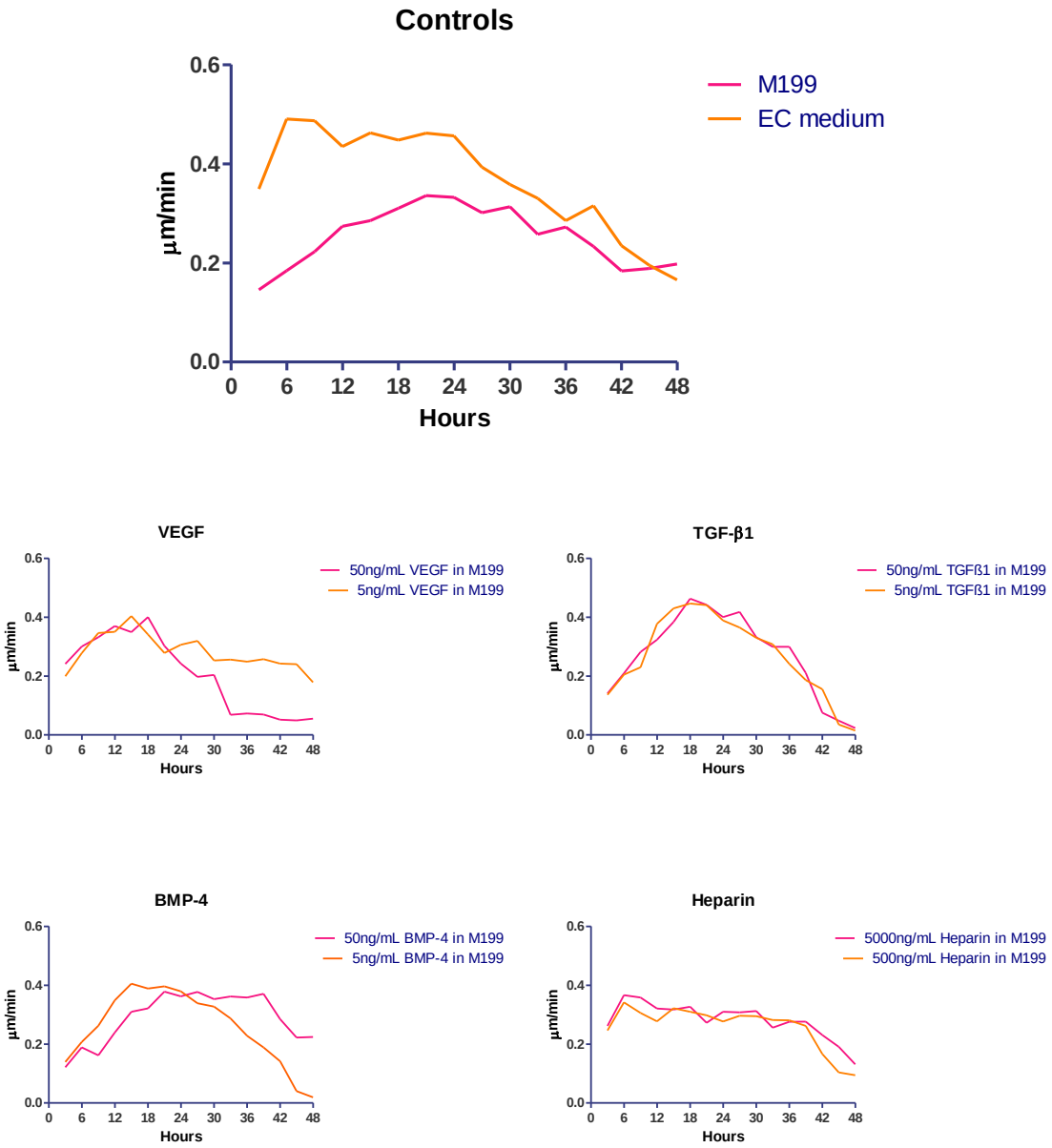



**Figure 59: 2D velocity of cells in response to stimuli** in M199 (basal) medium over 48 hours, shown by dosage. Normal EC medium is the positive control. Note difference in dosage scale for heparin. n = 3 wells per condition, 20 cells in each well measured for velocity over 3 hour time frames

Figure 59 shows the velocity of cells over a 48 hour period in response to added growth factors. All of these experiments were conducted in basal medium (M199) and EC medium, with added growth factors, although only the basal medium results are shown. This is because it was determined that the basal medium conditions were more representative of the growth factor effect without the background noise of the cocktail

of factors present in EC medium. This difference is shown in Figure 60. In basal medium, cells express a burst response of velocity in response to most added growth factors (Figures 49 B, C, D) except perhaps for heparin (Figure 59E), which is mostly a linear response. This burst effect is presumably because of the response time of signaling cascades regulating cell motility, followed by reduction in motility due to decreasing growth factor availability. As Figure 59 demonstrates peak motility for all factors in the 15-24 hour range, the peak motilities for the higher dose of each growth factor are compared in Figure 61. Although no growth factor increases 2D motility over the full EC medium control, 50 ng/mL TGFβ did significantly increase cell motility over the negative control (M199).

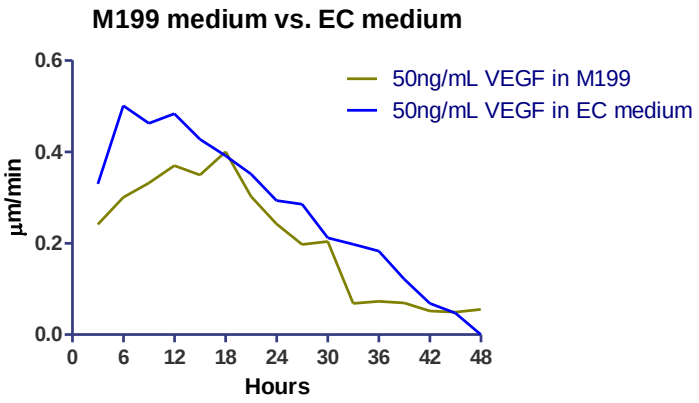



**Figure 60: Comparison of 2D cell motility over 48 hours** in response to 50ng/mL VEGF in M199 vs. in EC medium. n = 3 wells per condition, 20 cells in each well measured for velocity over 3 hour time frames

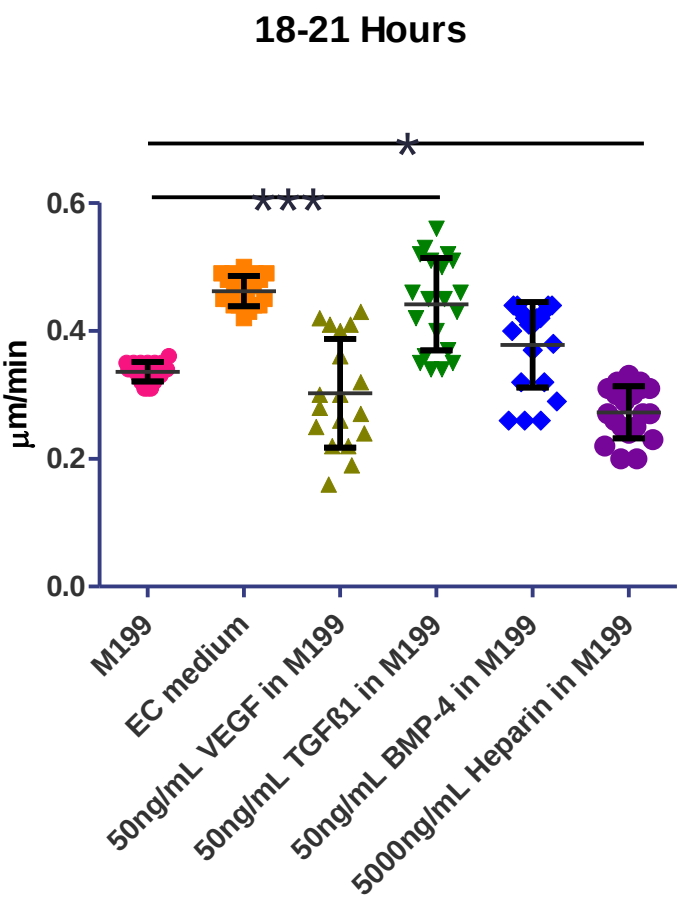



**Figure 61: Cell velocities measured over 18-21 hours after addition of stimulus** to the system. Full EC medium is the positive control. * = $p < 0.05$, *** = $p < 0.001$ n = 3 wells per condition, 20 cells in each well measured for velocity over 3 hour time frames

## DIVAA

The DIVAA, as purchased from Trevigen, was performed in CrTac: NCr-Foxn1nu homozygous mice from Taconic in accordance with NIDCR animal study protocol 10-571. Initial experiments were performed with negative (basement membrane extract [BME] and PBS) and positive (BME with VEGF and FGF-2) controls for 10 and 14 days in order to determine optimum experiment duration in the mice. Samples were then imaged and samples dissolved and quantification of FITC-conjugated Lectin determined through spectrophotometric immunohistochemical analysis using experimental reagents provided by Trevigen. The *Griffonia Simplicifolia* Lectin I provided in this kit is used for its ability to bind to the alpha-D-galactosyl and N-acetyl galactosaminyl groups

on the surface of endothelial cells. This specificity has been shown in the literature to be due to the specific tissue localization of these antigens on mouse endothelial tissues [394].

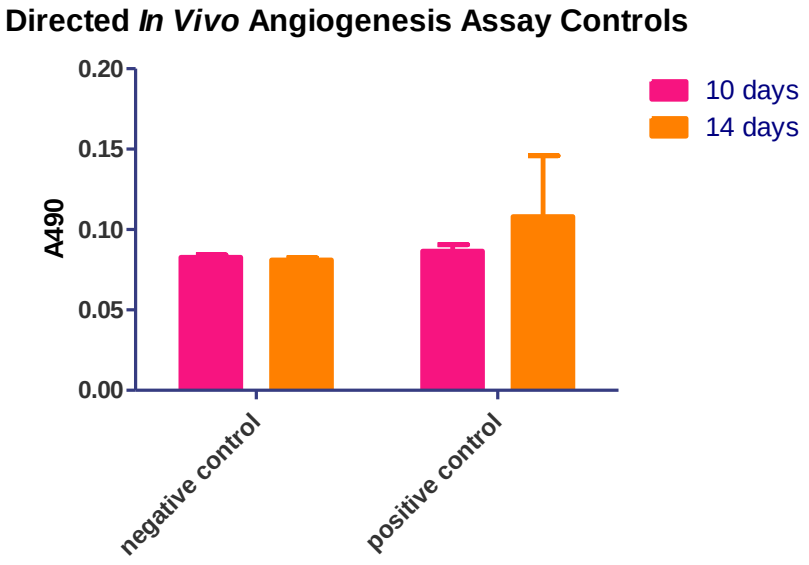
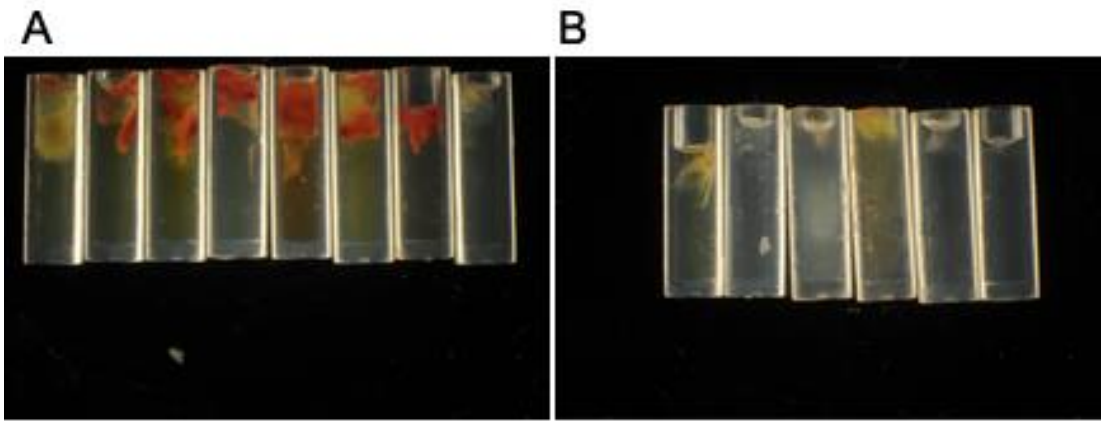



**Figure 62: Comparison of quantification of DIVAAs run with control conditions for 10 or 14 days** in order to determine optimum assay duration. n = 8 samples of each condition run in 2 mice with 4 transplants each. All transplants within each mouse were identical.

Our results (Figure 62) show no statistically significant difference between 10 and 14 days for either the negative or positive control. However, the amount of angiogenesis in the positive control was slightly higher after 14 days, which was used for the main experiment. The main DIVAA experiment consisted of positive and negative control samples, as well as two doses each of four different murine cytokines: VEGF, TGF-β, BMP-4, and heparin. These were analyzed for both vessel length (image analysis) and vessel quantity (as previously described).

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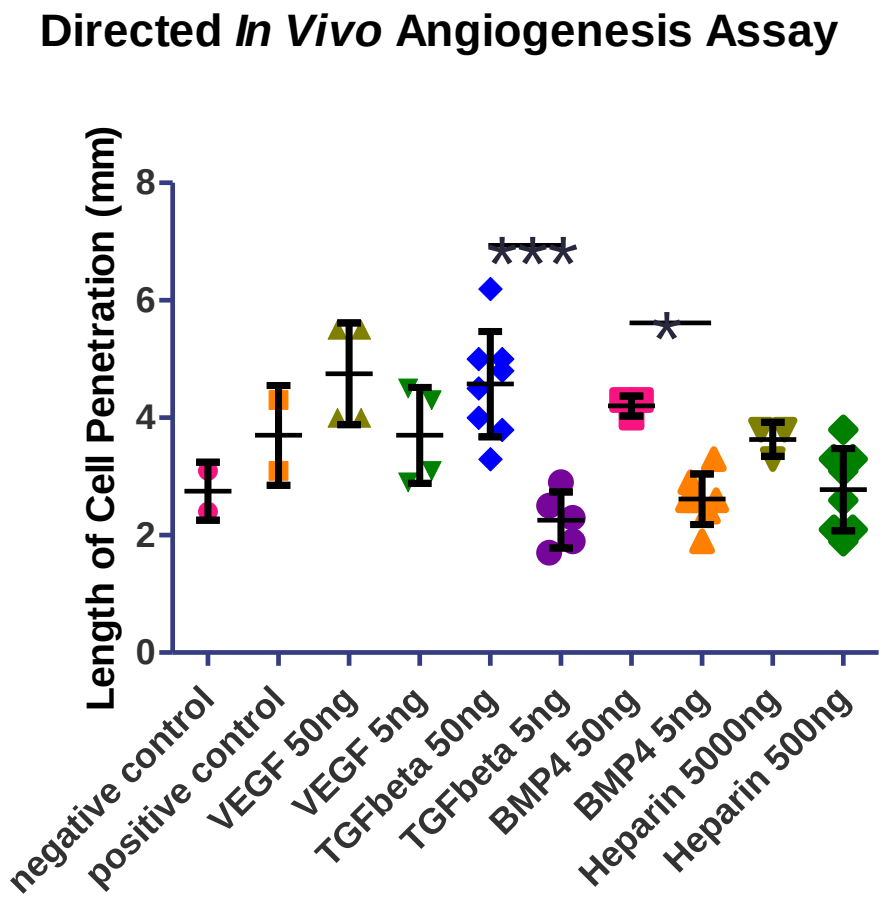


**Figure 63: Image examples of blood vessel penetration into transplants (A and B), and C) quantification of the lengths as measured through image analysis**. Images are of TGF-β at A) 50ng and B) 5ng doses after 14 days. * = $p < 0.05$, *** = $p < 0.001$ Original n = 8 samples of each condition run in 2 mice with 4 transplants each. All transplants within each mouse were identical. For the only 4 DIVAA reactors were transplanted, and 2 recovered. Transplants recovered: negative control = 2, positive control = 2, VEGF 50ng = 4, VEGF 5ng = 4, TGF-β 50ng = 8, TGF-β 50ng = 6, BMP4 50ng = 4, BMP4 5ng = 8, Heparin 5000ng = 4, Heparin 500ng = 8.

Figures 63 A and B are good examples of what the DIVAA system looks like upon harvesting. In addition to red blood vessels, there is also the infiltration of non-vessel-associated cells that might be ECs prior to vessel formation. Statistically significant differences are more difficult to obtain in this system due to the natural variability of *in vivo* systems. In figures 63C and 64, blood vessel length and number of blood vessels in the system are correlated. This is a crucial observation because it means that length of vessels is not gained at the expense of vessel number. However, the differences between the effects of various growth factors are more pronounced in the amount of lectin measured (representative of quantity of endothelial cells) as contrasted to the differences in vessel length between samples.

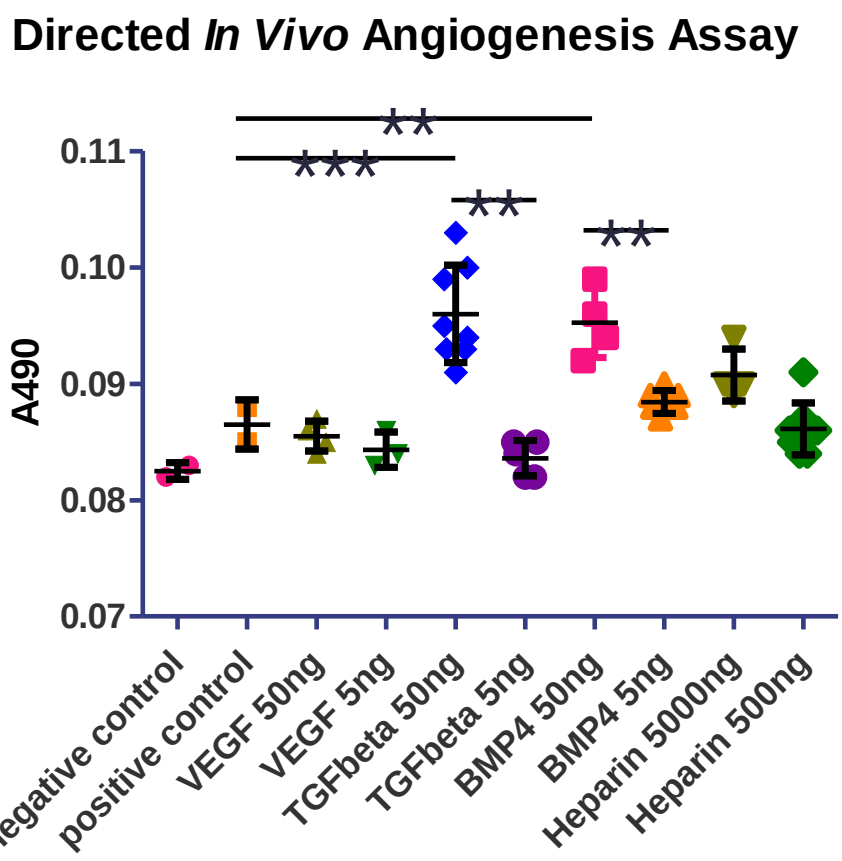


**Figure 64: Quantification through spectrophotometry of FITC-Lectin (at A490), indicating the numbers of ECs within the transplant system.** ** = $p < 0.01$, *** = $p < 0.001$ Original n = 8 samples of each condition run in 2 mice with 4 transplants each. All transplants within each mouse were identical. For the only 4 DIVAA reactors were transplanted, and 2 recovered. Transplants recovered: negative control = 2, positive control = 2, VEGF 50ng = 4, VEGF 5ng = 4, TGF-β 50ng = 8, TGF-β 50ng = 6, BMP4 50ng = 4, BMP4 5ng = 8, Heparin 5000ng = 4, Heparin 500ng = 8.

## Discussion

The transfer report for this project presented an extensive Boyden chamber assay studying the dose-dependent effect of a number of growth factors on cell migration (results not shown). However, it was argued at the time that the study did not conclusively show movement in response to a chemokine gradient as the existence of the gradient could not be proven through statistically significant results or the use of adequate controls. Therefore, when looking for a more reliable method for studying cell migration in response to added growth factors, two solutions were determined: first, a modified Boyden chamber assay, and secondly, a 2D motility assay.

The setup of a Boyden chamber assay is a tissue culture well with an insert suspended above; i.e., a well within a well (transwell) that has a membrane at its bottom with 8 μm pore size. HUVECs when rounded up have a mean cell diameter of 17 μm [289] and therefore, generally do not undergo mass movement across the membrane unless they are stimulated to do so. However, 8 μm is much larger than the size of any of the growth factors being studied, so the question becomes one of how fast the growth factor would equally distribute itself across the membrane. There is no physical osmotic pressure as, when following the manufacturer's instructions, specific volumes are placed on either side of the membrane. Still, the need for a chemical balance across the membrane will lead to some movement of the molecules across. In the new study, this eventuality was countered through two different methods. The first way this issue was dealt with was through the use of three controls: a) a negative control of basal (M199) medium on both sides of the membrane, b) a positive control of basal medium on top of the membrane (with the cells) and full (serum-containing) EC medium on the bottom, and c) a control for gradient equilibration by placing reduced-strength endothelial cell medium on both sides of the membrane. This random control gave a migratory result (shown in Figure 56) in the middle of, and significantly different from the positive and negative controls.

As expected, increasing the serum in the system increases overall movement compared to the negative control. Neither of these samples had a gradient; however, the addition of serum increased random movement, and therefore increased the number of cells that by chance went through the membrane over time. In contrast, the positive control, while containing the same amount of serum as the random control in the system, had this concentrated on the bottom of the membrane, creating a gradient. The result that more cells had gone through the membrane towards this source of stimulus over the course of 24 and 48 hours, as contrasted to the random control demonstrates the effect of a *sustained gradient*.

The next issue to discuss is the effect of time on this assay. This experiment had time points at 24 and 48 hours, in order to give the migrating HUVECs more time to process the signals coming from the bottom well. However, every single condition tested showed fewer cells on the bottom of the membrane at 48 hours than at 24 hours (hence the y-axis being half as high for the 48 hour results). There is no reason why the cells would migrate across the membrane at the start, and then migrate back towards the top well. Therefore, it must be concluded that the cells undergo apoptosis and/or fall off the membrane. HUVEC medium must be changed every two days, otherwise the cells undergo apoptosis. Under all conditions tested, the HUVECs are grown in diluted, depleted, or completely factor-free medium, an environment in which they are likely to struggle to survive, let alone grow. Thus, the 48 hour time point is less an indicator of stimulated movement (though it can still provide insight), but more an indicator of what conditions lend themselves towards HUVEC survival. In consequence, it is believed that the 24 hour timepoint is the best indicator of HUVEC response to stimulus.

Conditioned medium is, as previously discussed, both enriched with secreted growth factors from the cells that it was conditioned over, but also depleted from its original nutrient content by these same cells. Therefore, in order to maintain the same

level of motility induction as the control medium, the conditioning cells must add just as much motility stimulating substances, as they remove. This did not happen, as demonstrated by the results (Figure 57) indicating lower motility across the membrane for cells that were treated with conditioned medium versus the original full medium. However, the conditioning cells on balance, provided/left more stimulating signals than would be present in purely basal medium.

Placing cells in the bottom well of a Boyden Chamber Assay tests for a different type of motility induction than that which is tested through the use of conditioned medium. Conditioned medium tests induction of cells in response to the balance of factors taken up/released by other cells. Placing cells in the bottom of the system measures the motility induced by communication of cell types with each other, albeit in a non-contact system. Thus, the cells in the bottom well are able to change their factor secretion in response to signals from the cells migrating through the membrane – a feature not present in the conditioned cell model where the conditioning cells are merely responding to their surrounding medium.

Figure 58 lends itself towards a number of conclusions. The first of which is that the placement of hBMSCs in the bottom well actually decreases movement of HUVECs through the membrane. This could be due to either of two reasons: either the hBMSCs are producing factors which discourage HUVEC movement through the membrane, or the hBMSCs are removing factors from the solution that then slows HUVEC migration across the membrane. Here, the decreased migration supports our finding from the conditioned medium experiment, that hBMSCs indirectly cause a decrease in migration compared to an equivalent HUVEC sample (either conditioned or cells in the bottom well). However, this correlation does not help to discern the cause for this decrease.

The second conclusion to be drawn from the study pertains to the effect of HUVECs present in the bottom well on the migration of HUVEC through the membrane.

At 24 hours HUVECs in basal medium showed an increase over the basal medium control, whereas at 48 hours, the increase was shown only in the presence of full medium in the bottom well with HUVECs as contrasted to full medium alone. This finding is encouraging, and the cause of it is easier to hypothesize. At 24 hours, which, as mentioned above, is more likely to be an accurate measure of cell motility – HUVECs communicate with HUVECs in a non-contact fashion in order to stimulate HUVEC movement towards each other. This attractive movement in basal medium is significant when compared to basal medium (our base-line control). However, at 24 hours this movement stimulated by HUVECs in basal medium is significantly less than compared to the positive control (28 versus 65 cells on the bottom of the membrane per field of view). Our positive control is full endothelial cell medium. As this medium has been scientifically optimized over many years to aid the growth of HUVECs, it is unsurprising that this control should set a high standard for cell survival and growth. However, at the 48 hour time point HUVECs in EC medium demonstrated a stimulatory effect on HUVEC movement through the membrane, whereas HUVECs in basal medium did not. The conclusion drawn from this is that non-contact HUVEC communication can have a significant impact on cell movement through the membrane, even above and beyond the movement afforded by our positive control, when given enough time to effect this larger change (through more communication between cells due to increased cytokine excretion into the system). The result in basal medium of the loss of significant movement is not a measure of loss of stimulus, so much as one of lack of survival signals from the HUVEC in the bottom well, above that afforded by basal medium. The reason why the 48 hour result in full medium can be observed in the first place, is because the full medium in the bottom allows for enough cell survival in order for the HUVEC interaction to have an effect (although it must be noted that even in full medium, cell counts through the membrane decrease from 24 to 48 hours).

The purpose of the 2D motility assay is to measure random 2-directional movement of cells in response to growth factors added to the medium. HUVECs were plated at low density, and at time-point zero they were switched to growth factor-containing medium (either basal or full EC [only the former shown]). Samples were then placed in an incubated (temperature and $CO_2$ controlled) microscope, and imaged every 5 minutes for 48 hours. Essentially, this creates a scientist's version of a childhood comic flipbook, by moving through the images in sequence quickly, a video can be created and movement visualized and quantified.

All cultures demonstrated random non-directional movement, which is expected because no attempt was made to create a gradient. All cultures appeared to have peak velocity in the 15-24 hour time period (as shown in Figure 59), for both high and low doses of growth factors.

The 5 and 50 ng/mL doses for VEGF, TGF-β, and BMP-4 were chosen based on previous studies in the literature [262, 395]. Keogh *et al*. studied the effects of TGF-β application to cells on a 3D scaffold *in vitro* either at 0.2 ng/mL or at 10 ng/mL for the first 7 days (out of 49) followed by 0.2 ng/mL. They found increased changes in gene expression, mineralization, and other properties in the higher dose sample and cell infiltration into the scaffold was generally observed, but not specifically with regards to TGF-β dosage. Mishima *et al*. used a Boyden chamber assay to study chondrocyte migration and used 1 ng/mL and 10 ng/mL doses of TGF-β and 5 ng/mL and 50 ng/mL dosages of several other growth factors including VEGF. They did not find significant differences between the doses for VEGF or TGF-β. We chose to test 5 ng/mL and 50 ng/mL doses for our research as they appeared to be the industry norms.

The observed general decline in cell velocity after 36 hours (Figure 59) was due to widespread cell clumping and apoptosis after this period, primarily as HUVECs do not survive in basal medium, even mildly supplemented basal medium, for long periods of

time. We therefore chose to focus on cell velocity measurements taken between 18 and 21 hours after chemokine addition (Figure 61). Although none of the added growth factors increased cell velocity more than the positive control of full medium, TGF-β increased cell motility significantly over the negative control. Our results are an improvement over the findings of Mishima *et al*. [395], who found no difference between the negative control and TGF-β. BMP-4 treated cells had significantly less motility than the negative control. This does not, however, indicate that BMP-4 would not be appropriate for use in the transplant system, but simply that it does not increase random HUVEC motility *in vitro*. Similarly, VEGF and heparin, neither of which were significantly different from the negative control, might serve other purposes.

The DIVAA allowed for *in vivo* measurement of endogenous endothelial cell response to growth factors presented in a transplant system. Although this assay does not measure the bone and hematopoietic effect of adding these cytokines to a transplant, the system offers several advantages over our ectopic bone-forming model in terms of pure measurement of angiogenesis. As the name suggests, the blood vessel growth is directed. Since cells can only grow from one side of the tube, length penetrated can very easily be measured in a straight line. Also, the clear tube allows for straightforward imaging of the sample (Figure 63A and B), unlike the transplant, which would have to be sectioned and then analyzed. The third advantage of the DIVAA system is the uncomplicated, established method for dissolving the structure, whereas with a boney transplant decalcification of bone would be a necessary step, together with inclusion of methods to dissolve the scaffold.

As the DIVAA manufacturer recommended running the transplants for 8-14 days, we tested two different time points to measure basic performance. The results from our preliminary DIVAA tests indicated no significant difference between negative and positive controls as quantified (Figure 62), which could be partly due to the assay

being sensitive to air bubbles introduced during tube filling, inexact severance of the tube from the surrounding tissue during the tube extraction, and loss of material during flushing of the tube contents for quantification. However, it may be assumed that running the experiment for 14 days had the potential to increase blood vessel formation in our samples more than over 10 days, without overrunning the length of the transplant tube.

The final, main DIVAA also demonstrated no significant difference between positive and negative controls, either in length (Figure 63) or quantified ECs (Figure 64); however, a number of important observations can be made about the different growth factors tested and their dose dependent effects. In terms of vessel length, both TGF-β and BMP-4 demonstrated significantly deeper penetration of blood vessels into the transplants at 50 ng/transplant than at 5 ng/transplant. This dose-dependent response also was present when the ECs in the system were quantified. Additionally, both TGF-β and BMP-4 samples had a higher number of ECs present than the positive control (and negative control), making them the prominent EC migration inducers. The TGF-β results are in line with the 2-dimensional movement study results, where the 50 ng/mL TGF-β increased cell motility. However, in the 2D study, BMP-4 actually displayed a decrease in cell motility, which apparently does not correlate, in this instance, with decreased blood vessel formation *in vivo*. Arguably, blood vessel formation is a much more complex process than simple EC migration, as BMP-4 has been reported in the literature to be linked to VEGF-mediated angiogenesis [328, 329]. Neither VEGF nor heparin demonstrated direct stimulation of blood vessel ingrowth; yet they might aid blood vessel growth when combined with other factors or play other vital roles to support the process.

# Chapter 7: Conclusions and Future Work

The goal of this DPhil project was to study specific avenues to increase angiogenesis within the traditional paradigm of osteogenic tissue engineering. I have no doubt that tissue engineering is an approach that can become a transforming medicine of the future. Moreover, vasculature is the cornerstone of every tissue (other than cartilage). The formation and maintenance of a vasculature system within an osteogenic transplant has been, and will continue to be, a field of research for many laboratories throughout the world.

As discussed, the formation of bone within a tissue engineered construct follows the general bone formation pathways, but within a unique context. The formation of vasculature within this system is perhaps one of the most fascinating aspects of this process, and requires both angiogenesis and vasculogenesis. This subject was studied from both *in vivo* and *in vitro* approaches. The project design had the effect of showing true *in vivo* behavior as well as detailed experimental *in vitro* observation to hypothesize the processes behind the observed *in vivo* outcomes. In Chapter 2, I laid out 4 specific targets for this project, which I will address specifically here.

## Aim 1: Viability of a Syngeneic Mouse Model for Bone Formation

One of the larger breakthroughs of this project was the successful development of a syngeneic mouse model for the study of heterotopic bone formation in transplants, which showed that the model system worked in inbred FVB-N/J mice. Initial studies showed high scoring osteogenesis and hematopoiesis in transplants seeded with bone marrow stromal cells taken from nine individual mice. These experiments were followed by a study of pooled cells from five mice, which scored even better. It is perplexing that in our final study the positive control of cells pooled from five mice failed to perform adequately. These samples did produce extracellular matrix and some sort of fibrous tissue, but were not osteogenic or hematopoietic.

Another major advance of the project was the ability to successfully isolate and verify a CD31 positive, endothelial cell population from total bone marrow from mice. Human endothelial cells are widely commercially available, and even these are acknowledged to require complex medium and close attention. Mouse endothelial cells are known for being much more difficult to analyze and culture. Although a culturing method for bone marrow derived CD31 positive cells was not developed, a successful isolation protocol was devised to obtain the endothelial cell fraction in sufficient numbers through combining the marrow of a number of mice. Some researchers have suggested that a feeder layer of bone marrow stromal cells might be an appropriate solution, due to observed distribution of endothelial cells within an adherent bone marrow population *in vitro*.

## Aim 2: *In Vivo* Performance of Collagen-Based Materials

As mentioned in the Introduction, there are many different materials, ranging from biological materials, to demineralized tissues, through to completely synthetic scaffolds, which are being tested by various groups around the world. Composite materials are made in order to take advantage of the attributes of several different compounds. Some scientists will spend whole careers studying a single type of material. For the current project, a number of scaffold types were made and modified, which were predominantly studied for their osteoconductive potential in the context of various cell types, instead of focusing on pure materials characterization. The scaffolds used in this project were all collagen-based and completely biological. Most were crosslinked, either chemically (EDC-NHS), or physically (DHT), in order to improve their mechanical properties. When tested, it was found that crosslinking did not appear to have an effect on the histological performance of the transplant, either between crosslinking methods, or between crosslinked and un-crosslinked scaffolds. The identity of the material used did, however, have an effect, with Gelfoam™ performing better than our fabricated

collagen scaffold, which was, in turn, better than our fabricated collagen/hydroxyapatite composite scaffold. The underperformance of our synthesized scaffolds in comparison to Gelfoam™ occurred even after removal of the wall-like edges of the scaffolds, which had been discovered through use of SEM.

As a material, Gelfoam™ has been shown to be capable of forming high scoring bone and hematopoiesis in both immunocompromised as well as syngeneic mice. Although Gelfoam™ appears to be a very viable construct for mouse transplantation, with regards to the success of human osteoid growth, the material appears to be undesirable. Two hypotheses for lack of human osteoid formation are conjectured. Either Gelfoam™ and hBMSCs have a less successful relationship than Gelfoam™ and mBMSCs, in terms of physical structure, or chemical interaction; or some difference in the processes by which hBMSCs form bone, e.g. an excreted factor or ability to communicate, is less compatible with Gelfoam™. DHT crosslinking of the scaffold did not increase bone formation on the scaffold. Nevertheless, both experiments in this project, as well as previous work by collaborators indicate that Gelfoam™ seeded with human bone marrow stromal cells does in fact produce a very distinctive fibrous morphology, which is unfortunately unserviceable in attempts to form an osteogenic, hematopoietic transplant.

## Aim 3: Interaction of BMSCs and ECs in Co-Culture – Potential for Vasculogenesis

Scientists are constantly searching for ways to improve vascularization of transplants. The key to this problem in osteogenic transplants is the interaction between stromal cells and endothelial cells. This interaction was investigated in terms of the effect of osteogenic differentiation and changes in growth factor production. The ELISA assay results showed that while VEGF production by BMSCs was necessary for EC proliferation and governing of their behavior, production of VEGF by BMSCs was not

stimulated by culturing with ECs. On the other hand, BMSCs and ECs interact synergistically to produce elevated levels of TGF-β compared to either cell type. Such synergism is significant because TGF-β has been associated with EC motility, proliferation, and with vascular support functions. Previous work on the differentiation potential effect of adding HUVECs to hBMSCs had been contradictory. Some researchers claimed that co-culture inhibited osteogenic differentiation [389], whereas others noticed increased osteogenic differentiation [390, 391]. Our studies show a significant increase in calcification levels in mixed populations of stromal cells and endothelial cells as contrasted to either cell type alone. The effect of adding conditioned media is not as clear. In our larger, preliminary experiment, we found that conditioning the media by culturing over endothelial cells consistently increased the alizarin red S staining, i.e., calcification levels, of our samples. Conversely, in our second experiment, samples grown in conditioned medium decreased alizarin red S staining. The simplest explanation, in this case, is the most probable: not all batches of conditioned medium are the same. However, while alizarin red S results were suggestive of increased osteogenesis in HUVEC/BMSC co-cultures, these results were not corroborated by the results measuring the mRNA levels of osteogenic markers. These results further support the notion that *in vitro* assays may not reflect true osteogenic differentiation.

## Aim 4: Effects of Various Growth Factors on EC Motility – Potential for Angiogenesis

While studying the interaction of cells within the scaffold is crucial, interaction with the endogenous environment surrounding the transplant is also critical to understanding the nature of the system. As stated throughout this project, the goal was to stimulate both angiogenesis as well as vasculogenesis. In order to get nearby blood vessels to migrate into the scaffold endothelial cell migration toward the scaffold should be stimulated. The Boyden chamber assay attempted to determine the nature of

endothelial cell movement in response to either BMSCs or HUVECs through conditioned medium or non-contact cell communication. This new Boyden Chamber Assay has demonstrated several interesting properties of HUVEC migration.

- Conditioning medium with either HUVECs or hBMSCs decreases HUVEC movement
- Non-contact communication between hBMSCs and HUVECs
    - May decrease HUVEC movement through negative signals
    - Might simply decrease HUVEC movement by using up the stimulus in the system (artifact of the Boyden Chamber Assay model)
- Non-contact communication between HUVECs and HUVECs
    - Increases HUVEC movement towards each other
    - Does not increase HUVEC survival in a hostile (non-serum) environment

While these findings are very informative, the conditioned medium model poses questions of depleted medium – different cells will deplete the medium in ways that cannot be controlled for (adding arbitrary amounts of serum to the system will not solve this issue). Additionally, the 48 hour time point demonstrates the limitations of the Boyden Chamber Assay, because HUVECs will not survive for more than 48 hours in full EC medium (normal culture conditions), and survival rates in depleted or basal medium are significantly decreased after 24 hours, so the desire to perform longer time points in order to see a larger effect cannot be satisfied. It could be argued that even at the 24 hour time point, some of the difference between the random control and the negative control could be attributed to early cell apoptosis in the negative control (basal medium). While above it was stated that these problems reflect the limitations of a Boyden Chamber Assay, they really reflect the limitations of the scope of all *in vitro* assays. *In vivo* assays would provide self-replenishing physiological surroundings for

the HUVECs, thereby increasing their survival and demonstrating their true interactions with surrounding cells.

The premise of studying the effect of various growth factors on 2D motility of cells was the assumption that increasing the velocity of cells would increase movement into the scaffold, more so even if the random 2D movement could be translated into chemotaxis through the creation of a gradient from a soaked sponge in future applications. The experiment demonstrated a burst of velocity in response to several growth factors over time for all growth factors except for heparin, which demonstrated a slower decay. The nature of this burst response indicates, that, if this peak velocity were to be maintained, fresh growth factor would have to be added on a 24-hour cycle – easy to do *in vitro*, but not a viable solution for *in vivo* studies or clinical situations. Future studies would be necessary to determine if this burst effect of applied growth factor would be effective in an *in vivo* setting. Nevertheless, the burst kinetics of this study, show that these factors do indeed have an effect on endothelial cell motility, particularly TGF-β.

One of the most elegant studies in the project was the DIVAA. While only 2 weeks in duration, as contrasted to the longer 12-week transplant studies, the DIVAA provides a unique insight into the specific effects of each growth factor individually, at various doses, on endothelial cell growth into the transplant and the formation of structured blood vessels. It was found that both VEGF and TGF-β at 50ng/mL doses significantly improved blood vessel penetration into the system. In the case of TGF-β, an increase in penetration in the system correlated with increased motility observed in our 2D motility assay. VEGF did not significantly increase 2D motility, but it improved *in vivo* EC penetration, indicating that *in vitro* and *in vivo* performances are not always correlated. Notably, this experiment demonstrates that it takes the appropriate experiment to find the purpose of a particular factor. For example, VEGF did not

increase motility but was necessary for other biological processes related to blood vessel formation, as shown by the DIVAA results. Even if a growth factor is not significantly different from the controls in one assay type; that does not mean the factor does not serve a vital purpose in movement towards a desired outcome.

## Final Summary

This DPhil project encompassed not one, but several aspects of tissue engineering. Instead of focusing solely on materials, cells, or growth factors, I chose to study all three and how each aspect can be improved to better the performance of the scaffold. I particularly focused on angiogenesis due to its remaining elusiveness yet critical importance in tissue engineered constructs. In order to study these components, I developed a mouse model for osteogenic tissue engineering in syngeneic, immunocompetent mice, and a way of isolating endothelial cells from the bone marrow of these mice. Amongst the materials studied, Gelfoam™ performed the best in the mouse studies, but an alternative must be found with regards to human transplants. Studying the interaction of bone marrow stromal cells with both seeded endothelial cells as well as the endothelial cells in the surrounding tissue through a number of *in vitro* studies has been enlightening in the search for improved angiogenesis and vasculogenesis in tissue engineered transplants for bone regeneration.

## Future Work

Some of the future work conceived while writing this thesis has already been carried out in the time between thesis submission and thesis defense, and/or is currently still being investigated. The results from this short time frame are reflected in Appendix 6. Briefly, if this research were to continue, there are several avenues that should be pursued.

First, while the syngeneic mouse model is a promising breakthrough as a pseudo-autogenic model for bone transplantation in mice, it has so far only been tested in highly inbred FVB mice. This model could be expanded to test the limits of such transplantation as different mice have different bone regeneration potential due to their limited CFE (colony-forming efficiency) and/or due to immunogenicity due to antigens present when donor and host mice are not as closely related. Additionally, the effect observed that pooled cells actually perform better than an average of individual donors is a special phenomenon that requires both verification, and examination into the possible causes of this effect. This could be done with repetition of the experiment, combined with primary CFE analysis of these cell populations (Appendix 6), and should also be carried out with higher n numbers. A more conclusive test would be to test BMSCs from the same 5 mice: with some of the primary bone marrow cell population pooled for CFE, some kept for individual CFE – and both pooled and individual cell populations tested in the syngeneic mouse ectopic ossicle model. However, this latter experiment is severely limited by the number of cells that can be flushed from individual mice, and the need to keep cells at low passage for transplantation.

Another experiment that has been pursued (Appendix 6) but should be further investigated is the *in vitro* osteogenic assay. Both in the thesis and in appendices, it is discussed how this model is questionable in its validity (if only alizarin red staining is used as an indicator) and how this needs to be backed up by qPCR and other validating studies of osteogenesis. Originally, it was thought that the dystrophic calcification believed to be observed in the thesis was due to the β-GP in the osteogenic factor cocktail, but this has been called into question by the results from Appendix 6 (at least, β-GP does not appear to drive dystrophic calcification under low-serum conditions). Therefore, if the true nature of this experiment is to be understood, the various components need to be isolated and studied. Also, more time points are necessary in order to measure the osteogenic progression not just the end point.

Chapter 4 interestingly pointed out the limitations of Gelfoam™ as a scaffold material. Something about the human cell interaction with the material is crucially different from the mouse cell-scaffold interaction, resulting in the inconclusive histology observed. First, this experiment should be repeated with the hBMSCs on a known positive control product as well as the Gelfoam™. But also, *in vitro* studies could measure survival rates of the cells on the scaffold. Additionally, 2-D studies to test cell adherence to the scaffold may be called for. If no difference is found in these studies, then changes in gene expression pre- and post-adhesion to the Gelfoam™ and control scaffolds should be tested.

With regards to results from the preliminary experiments, it could be argued that the effect of channels in the transplant should be further studied in a mouse strain where the positive control forms bone and hematopoiesis. The experiment was performed in the NIH III nude mouse prior to our diagnosis of that strain's genetic drift, and therefore was perhaps unfairly discarded and not pursued further.

Perhaps the most elegant experiment presented in this thesis was the DIVAA. This system has the potential to test combinations of growth factors, similar to the ones individually tested in the thesis, and might even be able to demonstrate spatial effects, through the layers of different growth factors in the BME in bands at varying lengths from the aperture.

Importantly, ultimately, growth factors on scaffolds with or without cells will need to be tested in order to observe the outcomes of a seemingly infinite combination of factors. However, the growth factor kinetics in the system should be considered, and with further study, might eventually be modified either through the application of transfected cells which inducibly/constitutively over-express desired factors, or through the addition of time-release systems for stimulus.

Whichever future experiments are pursued, it will always be crucial to keep the successful stimulus of vasculature formation (both through vasculogenesis and through angiogenesis) as a primary goal – in order to demonstrate that increasing vasculature will therefore improve transplant performance *in vivo*.

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# Appendix 1: Materials and Machines

## Scaffold Creation

Collagen: Collagen from bovine Achilles tendon, type I, from Sigma, # C9879-10G

Hydroxyapatite: as made by self

Gelfoam™: Gelfoam™ 12x7cm, from Pharmacia and Upjohn, #6510-00-080-2053

## Cell Culture

hBMSCs: primary culture from a patient sample, obtained from Dr. Marianna Sabatino at the NIH Clinical Center

mBMSCs: primary culture self-derived from mice

HUVECs for ELISA: primary culture derived by the laboratory of Dr. Tosato, National Cancer Institute at the NIH

HUVECs all other experiments: HUVEC-Umbilical Vein, Pooled Cells, EGM-2, cryo-ampule, from Lonza, #C2519A

Cytometer: Cellometer Auto T4 by Nexelcom Biosciences

αMEM: αMEM, from Invitrogen, #12571071

M199: CellGro Medium 199, from Corning, #10-060-CV

Fetal Bovine Serum: Fetal Bovine Serum, from Atlanta Biologicals, # 11150

Human Serum: Human Serum, from Sigma Aldrich, #H4522

Newborn Calf Serum: Newborn Calf Serum, from Sigma Aldrich, #N4637

Gelatin: Bovine skin gelatin, from Sigma Aldrich, #G9391

Pen-Strep: Pen-Strep, from Invitrogen, #15140122

GlutaMAX™: GlutaMAX™, from Invitrogen, #35050061

ECGS: Endothelial Cell Growth Supplement, from Sigma Aldrich, #E2759

Heparin: Heparin sodium salt, grade 1-A from porcine intestinal mucosa 170units/mg, from Sigma Aldrich, #H3393-250KU

Ascorbic Acid: L-ascorbic acid phosphate magnesium salt hydrate, from Waco, #013-12061

Trypsin: Trypsin, from Invitrogen, #25300120

## IHC

αSMA antibody: Rb anti αSMA, from Abcam, ab5694

Alexafluor: Alexafluor 488 goat anti rabbit IgG, from Invitrogen, #a11008

Antibody diluent: Antibody diluent, from Zymed, #00-3118

Mounting medium: Mounting medium with DAPI, from Vectashield, #H-1200

## FACs and MACs

MACs column: LS column, from Miltenyi, #130-042-401

Anti-CD31 antibody: anti mCD31, from R&D Systems, #BAF3628

Anti-biotin beads: anti-biotin microbeads, from Miltenyi, #28419

Streptavidin beads: streptavidin microbeads, from Miltenyi, #20221

Cd31 FACs Ab: APC Rat anti-mouse CD31, from BD Pharmingen, #551262

Cytofix: Cytofix fixation buffer, from BD Pharmingen, #554655

Stain Buffer: Stain buffer, from BD Pharmingen, #554657

## Osteogenesis Assay

Dexamethasone: Dexamethasone, from Sigma, #D-4902

β-glycerol phosphate: β-glycerol phosphate, from Sigma, #G4222-50g

Ascorbic acid phosphate: see above

Alizarin Red: Alizarin red S, from Sigma-Aldrich, #A5533-25G

Shredder: QiaShredder, from Qiagen, #79364

RNA prep: RNAeasy, from Qiagen, #74104

cDNA prep: iScript cDNA synthesis kit, from BioRad, #170-8891

Sybr green: iQ™ Sybr Green Supermix, from BioRad, #170-8882

qPCR machine: BioRad CFX96 real time system with a C1000 thermocycler

## ELISA

VEGF ELISA: VEGF DuoSet, from R&D Systems, #DX293B

TGF-β ELISA: TGF-β DuoSet, from R&D Systems, #DY240

## 2D motility

TGF-β: rhTGF-β1, from R&D Systems, #240-B/C

VEGF: rmVEGF, from R&D Systems, #493-MV/CF

BMP4: rmBMP-4, from R&D Systems, #5020-BP/CF

Heparin: see above

## Boyden Assay

Assay kit: HTS Fluoroblock™ multiwall insert system, from BD Falcon, #351158

Mounting medium:  Prolong gold anti-fade reagent, from Invitrogen, #p36930

## DIVAA

DIVAA kit:  Starter Kit, from Trevigen, #3450—48-SK

TGF-β:  rhTGFbeta1, from R&D Systems, #100-B/CF

VEGF:  rhVEGF, from R&D Systems, #293-VE/CF

BMP4:  rhBMP-4, from R&D Systems, #314-BP/CF

Heparin:  see above

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# Appendix 3: Academic CV

**Nolanne Arlette Chang**

(Full legal name: Nolanne Arlette Chang-Wai-Ling)

***EDUCATION:***

2008-present **DPhil** in Orthopaedics, Rheumatology, and Musculoskeletal Sciences

- *Oxford University, Wolfson College*
- Sponsored by, and in collaboration with, the National Institutes of Health (Bethesda, USA)
- Joint with the National Science Foundation Graduate Research Fellowship Program
- Advisors: Prof. James Triffitt, Dr. Jan Czernuszka, Prof. Udo Oppermann, Dr. James Dunford, Dr. Kenn Holmbeck, Dr. Pamela Gehron Robey

2004-2008 **Masters** in Molecular and Cellular Biochemistry

- *Oxford University, Somerville College*
- Undergraduate masters degree (honours) including a Masters research project and dissertation joint between the Nuffield Department of Orthopaedic Surgery and the Materials Department

***RESEARCH EXPERIENCE:***

2008-present **DPhil**: Towards the Development of Vascularized Constructs for Bone Repair

- *National Institutes of Health* (Bethesda, MD, USA)
  - National Institute of Dental and Craniofacial Research, Craniofacial and Skeletal Diseases Branch (Dr. Pamela Robey and Dr. Kenn Holmbeck)
- *Oxford University* (Oxford, UK)
  - Nuffield Department of Orthopaedics, Rheumatology, and Musculoskeletal Sciences (Prof. James Triffitt, Prof. Udo Oppermann, and Dr. James Dunford)
  - Department of Materials (Dr. Jan Czernuszka)

2007-2008 **Masters Research Project and Dissertation**: The Incorporation of Heparin into Tissue Engineering Scaffolds and the Effect on Musculoskeletal Progenitor Cells

- *Oxford University* (Oxford, UK)
  - Department of Materials (Dr. Jan Czernuszka)
  - Nuffield Department of Orthopaedic Surgery (Prof. James Triffitt)

2004-2006 (Vacations) **Research student intern**

- *Sandia National Laboratories* (Albuquerque, NM, USA)
- Organization 1114: Surface and Interface Science (Dr. Julia Hsu)
- Developing a sol-gel printing system for creating semi-conductors on the micro- and nano-scale
- Developing a novel three-dimensional composite solar cell system

***HONORS and AWARDS:***

2008-2011 Winner of a National Science Foundation Graduate Research Fellowship based upon a successful research proposal

***ABSTRACTS/PUBLICATIONS***

**Publication:**

Chang, Nolanne A.**,** Richardson, Jacob J., Clem, Paul G., and Julia W. P. Hsu. **Additive Patterning of Conductors and Super-Conductors by Solution Stamping Nanolithography** Publication in ***Small***, vol. 2, issue 1, 2005: pp. 75-79

**Presentations:**

NIH-OxCam Colloquium, June 2013: **Introduction to a Syngeneic Mouse Model for the Study of Bone Transplants**. Nolanne Chang[1,2], Kenn Holmbeck[2], Pamela Gehron Robey[2]; [1]University of Oxford Nuffield Department of Orthopaedics, Rheumatology, and Musculoskeletal Sciences, [2]Craniofacial and Skeletal Diseases Branch, NIDCR, NIH, DHHS

Materials Research Society Spring Meeting 2006: **Structure Control in ZnO/Polymer Composite Solar Cells.** Thomas L. Sounart[1], Erik D. Spoerke[1], Dana C. Olson[2], David Scrymgeour[1], Nolanne A. Chang[1], Terry R. Guilinger[1], Sean E. Shaheen[2], Julia W.P. Hsu[1], David S. Ginley[2] and James A. Voigt[1]; [1]Sandia National Laboratories, Albuquerque, New Mexico; [2]National Renewable Energy Laboratory, Golden, Colorado.

Materials Research Society Fall Meeting 2004: **Solution Stamping Nano-Lithography for Patterned Deposition of Inorganic Films.** Chang, Nolanne A., Hsu, Julia W.P., Richardson, Jacob J., and Clem, Paul G; Sandia National Laboratories, Albuquerque, New Mexico.

**Posters:**

TERMIS-NA (Tissue Engineering & Regenerative Medicine International Society – North America) Conference, December 2011: **Optimizing Components of an Angiogenic Scaffold System for Bone Regeneration.** Nolanne Chang, Kenn Holmbeck, Pamela Gehron Robey

TERMIS-NA (Tissue Engineering & Regenerative Medicine International Society – North America) Conference, December 2010: **Scaffold Materials and Design and Cell Choice for Bone Regeneration Transplants.** Nolanne Chang, Brian Sworder, Asma Yahyouche, ; Li Li, Jim Triffitt, Jan Czernuszka, Pamela Gehron Robey

NIH-OxCam Colloquium, June 2010: **Development of a Vascularized Tissue Engineered Construct for Bone Regeneration using an *In Vivo* Model**. Nolanne Chang, Brian Sworder, Asma Yayouche, Li Li, Jan Czernuszka, Jim Triffitt, Pamela Robey

bone-tec International Bone Tissue Engineering Conference, October 2009: **Scaffold Design and Cell Choice for Bone Regeneration Transplants**. Nolanne Chang, Sayuri Yoshizawa, Natasha Cherman, Asma Yahyouche, James T. Triffitt, Jan T. Czernuszka, Pamela G. Robey

NIH-OxCam Colloquium, June 2009: **Construction of a Vascularized Bone Regeneration System**. Nolanne Chang, Brian Sworder, Sergei Kuznetsov, Zhidao Xia, Jan Czernuszka, James Triffitt, Pamela Robey

NIDCR Fellows Retreat, April 2009: **Incorporation of Heparin into Tissue Engineering Scaffolds and the Effect on Musculoskeletal Progenitor Cells.** Nolanne Chang, Zhidao Xia, Yuan-Tsan Tseng, Pamela Robey, James Triffitt, Jan Czernuszka

**Planned Publications:**

A Syngeneic Mouse Model for the Study of Bone Regeneration in FVB-N/J Mice.

Studies of Growth Factor Application on HUVEC Motility.

Note: CRL-11882, CRL-2496, and Saka Osteogenic Cell Lines Do Not Form Bone and Hematopoiesis *in Vivo*.

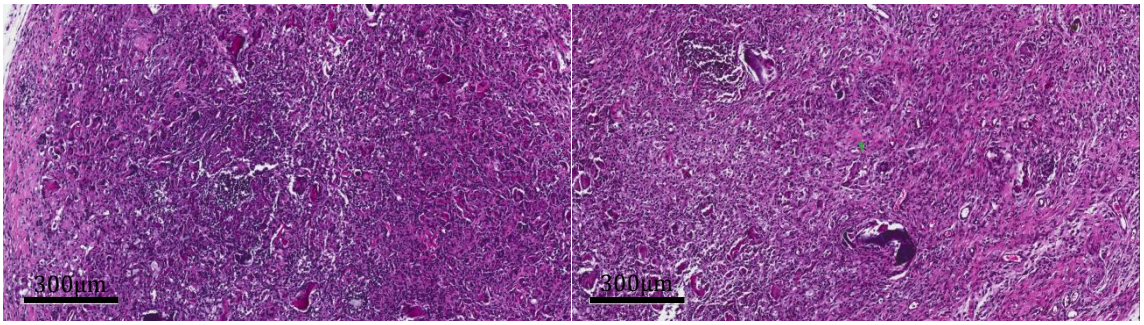
# Appendix 4: Transfer Report Findings – Preliminary Results

At a few different points in my thesis, but primarily in Chapter 2, I make reference to my transfer report. Rather than attach my entire transfer report, I thought it would be appropriate to place in an appendix the lessons learned from the transfer report experiments, and supporting figures.

Preliminary experiments were *in vivo* transplant experiments that were carried out in NIH III Nude mice for 8 and 12 weeks. Materials were tested either by themselves, without any seeded cells, or with bone marrow stromal cells and/or endothelial cells of human origin.

The first lesson learned from our preliminary experiments is that endothelial cells (EC) in and of themselves, when seeded without any other cell type, do not form tissue structure and matrix. This claim is made based upon observation of histology when sections were stained with H&E. As one can see from the images shown below in Figure A4-1, while none of the samples formed bone, the hBMSC-seeded and mixture-seeded scaffolds did form some sort of tissue structure, whereas the EC seeded scaffold merely resembled cells on a scaffold, despite having been *in vivo* for 12 weeks.

A) B)



C)

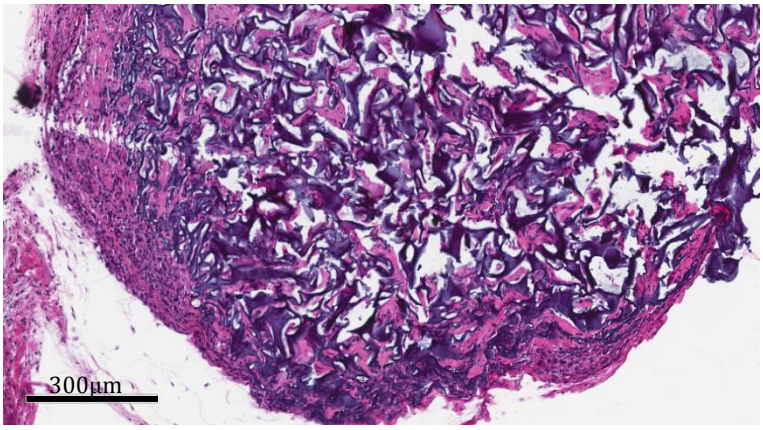



**Figure A4-1: H&E staining of preliminary transplants on collagen/hydroxyapatite composite.** A) seeded with hBMSC, B) seeded with hBMSC/HUVEC in a 50/50 ratio, C) seeded with HUVEC. Note that A and B demonstrate tissue formation, whereas C does not. All images are 10x magnification.

While all of my scaffold materials are porous throughout, one of the questions we attempted to answer in our preliminary experiments was whether the introduction of channels during the synthesis process could improve *in vivo* angiogenesis performance. In Chapter 4 of my thesis I quantify the amount of angiogenesis in a scaffold using fluorescent immunohistochemistry for alpha smooth muscle actin (αSMA), a marker found expressed on cells lining blood vessels. In the preliminary experiments I also used αSMA staining to quantify angiogenesis, but then I used a chromogenic stain. However, the rest of the process is the same, with the slide being imaged and quantified using ImageJ. For this experiment, we transplanted scaffolds with either no channels (remember, these scaffolds are still naturally porous), 2 small channels, or 1 large channel – as made by a collaborator in the Materials Department at the University of Oxford. The volume taken up by the scaffold was the same. These scaffolds had been made using the same technique of collagen suspension in aqueous solution, followed by freezing and lyophilization, but were made in special molds which had prongs running lengthwise through the cylinder. Once the mold was removed, this resulted in channel spaces with no collagen where the prongs had been.

A) B) C)

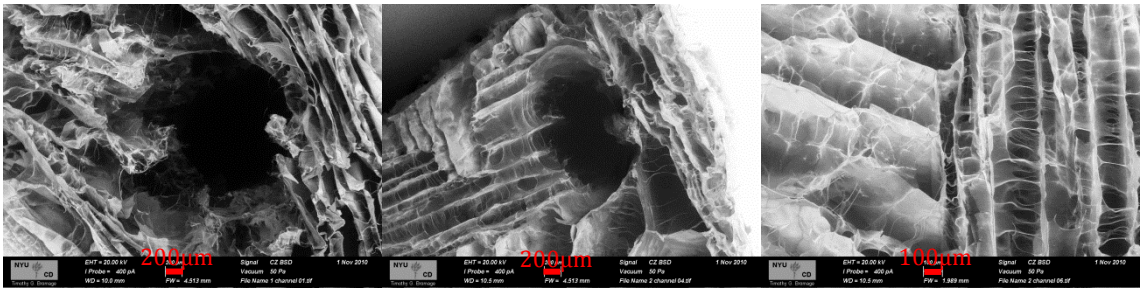



**Figure A4-2: Micrographs of** A) transplant with large hole B) transplant with small hole C) cross-section through hole from a transplant with small holes

The large channel had a diameter of approximately 2.0 mm, whereas the small channels measured approximately 1.2 mm in diameter. Thus, these channels are much larger than the pores in the surrounding material. These scaffolds with channels, along with the no channel controls supplied by the collaborator from the same batch of collagen suspension, had not been cross-linked in any way.

Our results from the *in vivo* transplantation, shown in Figure A4-3, demonstrate no difference in amount of vasculature formed between samples of with channels of different morphologies or samples with no channels at all.

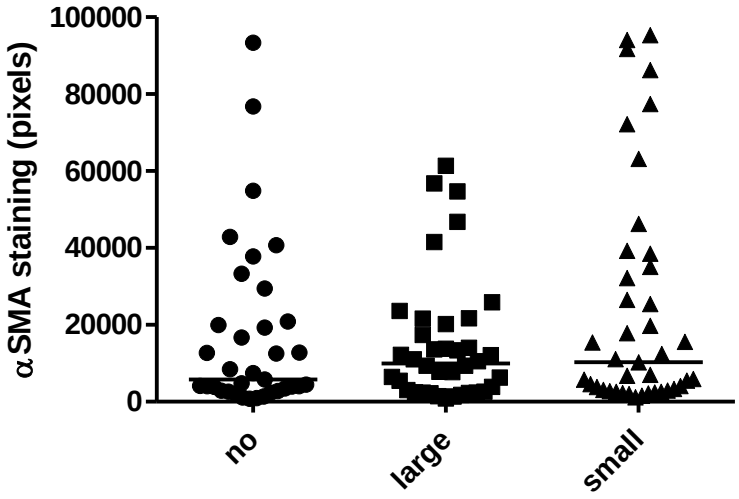



**Figure A4-3: Angiogenesis in porous scaffolds** with either no channels, one large channel, or 2 small channels. Note there is no significant difference in angiogenesis as measured by chromogenic αSMA staining. n = 3 samples transplanted per condition, each material was transplanted in triplicated with either

hBMSCs, HUVECs, or a combination thereof on them, but no difference was found between cell population results either, each transplant was stained via IHC on 3 slides, and 2 sections per slide were analyzed for αSMA quantification.

It is important to note that when placing the scaffolds in the ectopic mouse ossicle model, the orientation of the channels relative to the host could not be controlled, e.g. parallel to or perpendicular to the main body contour of the mouse. Additionally, no bone was formed in these or any other ectopic mouse transplants being attempted at the time – this was in the NIH III nude mice (discussed further below), and therefore it is unknown how much the histology of these transplants would have changed if this had, for example, been later tested using our syngeneic mouse model.

Another lesson learned is that crosslinking methods did not change scaffold performance, but material choice did. In Chapter 3, I mention, and show images of, the residue I found on the surface of EDC-NHS crosslinked scaffolds. While this is reason to have concern about EDC-NHS scaffolds, our preliminary findings from our *in vivo* transplants, showed no difference in performance between EDC-NHS and DHT crosslinked scaffolds in terms of the amount of angiogenesis, and therefore we were content not to pursue EDC-NHS crosslinked scaffolds any further. The results from this experiment are shown below in Figure A4-4. Nevertheless, we found that the choice of materials had a significant effect on angiogenesis, although specific material choice would require further experimentation.

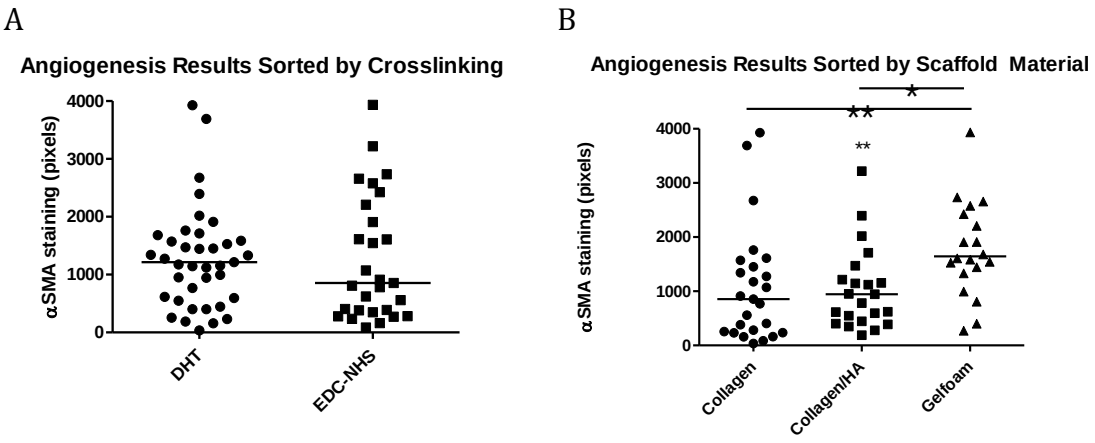



**Figure A4-4: In transplants that hadn't been seeded with any cells, angiogenic performance did not vary between crosslinking methods, but did differ between materials used.** This was our first indication to focus on Gelfoam™ as a transplant material. n = 3 for each material and crosslinking method combination, but for statistical purposes, scaffolds were grouped by either material or crosslinking, not kept discrete. Each transplant was stained via IHC on 3 slides, and 2 sections per slide were analyzed for αSMA quantification.

The final take-home message from our preliminary experiments was that the most significant difference was the difference between host mice as an effect on angiogenesis (Figure A4-5). This finding was highly problematic and unexpected, since all transplants within each experiment were performed at the same time, on mice the same strain and age, ordered from the same vendor at the same time. Clearly, a better model would have to be found.

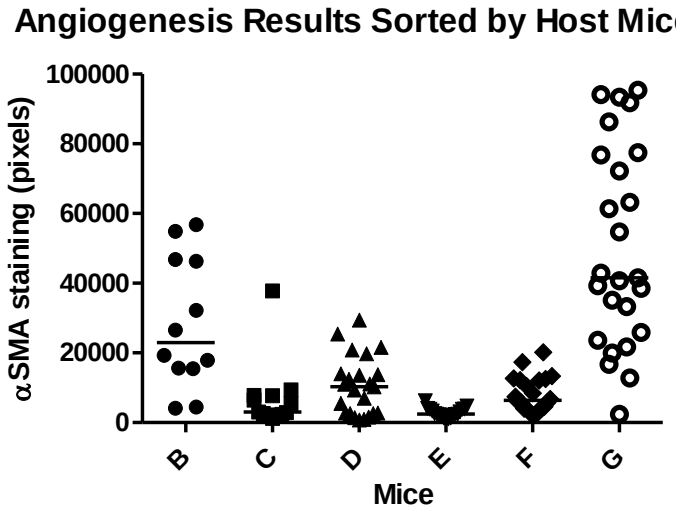



**Figure A4-5: Difference between angiogenic performance of transplants between host mice** – shown from the channel experiment**.** The difference between mice was very significant ($p < 0.001$). n = 6 transplants per mouse, each IHC stained analyzed on triplicate slides, 2 sections per slide.

# Appendix 5: Cloning Experiments

## Introduction

Utilizing growth factors in a tissue engineered construct system has long been studied in order to increase the biologically osteoinductive inductive properties of the transplant. There are several different ways of applying the growth factors to the system – listed here in order of increasing effect:

- Systemic injection of the growth factor at a site not related to the transplant
- Bolus injection of growth factor at the site of desired regeneration, in the absence of a physical transplant
- Soaking of the scaffold in a solution of growth factor prior to transplantation
- Immobilization/tethering of the growth factor to the scaffold via chemical or physical methods
- The seeding of cells onto the scaffold that overexpress growth factors (either inducibly or constitutively)

Systemic injection generally requires a much higher dose of growth factor, presents a much shorter half-life of the factor, and even a small degree of non-specific homing can have deleterious effects on other regions of the patient. Local application of the growth factor is a much more sensible solution, particularly in the context of a scaffold-system, which increases the half-life of the growth factor and controls release. The most powerful solution to this desire to increase local growth factor expression is to transfect cells to constitutively or inducibly over-express the desired stimuli. In order to test the hypothesis that increasing vascularization achieves better transplant outcomes *in vivo*, it was decided to transfect hBMSCs to over-express VEGF and TGF-β, because both of these growth factors have been linked to angiogenesis, vasculogenesis, and osteogenesis.

Cloning experiments were performed in two different sets. The first attempt to create cells that over-express these growth factors was carried out at the University of Oxford using the TetON system (Clontech: Mountain View, CA). After this set of cloning was deemed to have not produced any successful clones, a second set of cloning was undertaken at the NIH. In this second set, viral transfection (either adeno- or lenti-) was proposed to create the desired cells. However, after the final gene products were created, this project was halted due to observed difficulties when transfecting the desired hBMSCs. In this appendix, both sets of cloning experiments will be outlined, along with *in vivo* transplantation results of the cell lines used in the first set of cloning.

## Cloning Set 1 – University of Oxford

The TetON system is a two-part system to create transfected cells – and therefore it consists of two main stages to create the transfected cells. Both of these steps are verified by antibiotic resistance to select for clones that had incorporated the gene sequence. The first of these steps is the creation of a TetON clone that contains the inducible promoter. G418 was used to select for stably transfected cells, since it carried a G418-resistance gene. With cloning via this method it is unknown how strongly each cell expresses the new loci implanted (if more than one copy is inserted). Thus, once TetON clones are isolated and grown, some of each colony is transfected with the pTreTight-GFP gene that targets the TetON loci – providing a visual confirmation of TetON vector insertion and the strength of gene expression (when stimulated by Dox).

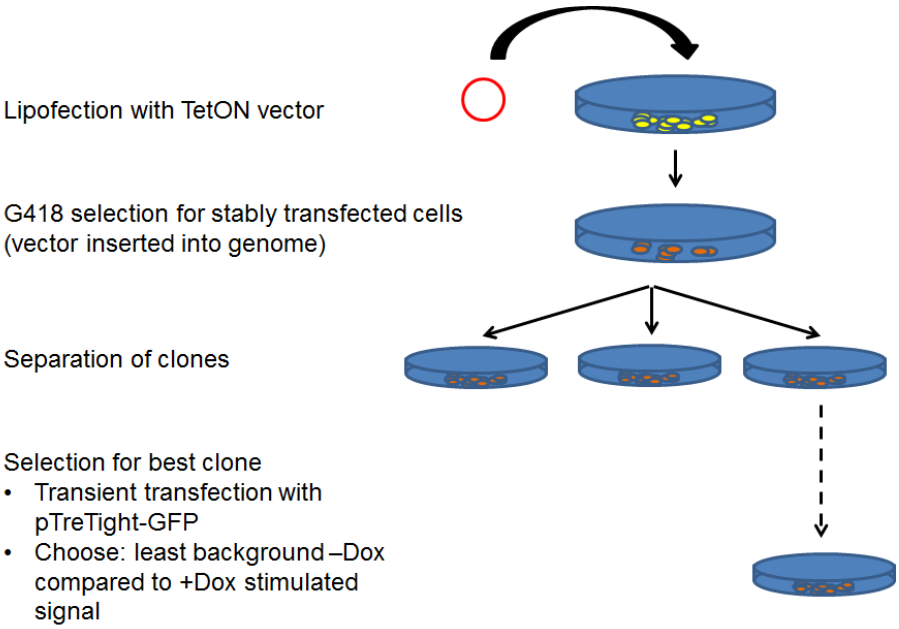



**Figure 5-1: Creation of a TetON clone**

The second stage is to insert the gene of interest (GOI) into the genome under the regulation of the TetON promoter. In order to perform this section of the process, vectors containing the GOIs were purchased, the GOI sequences amplified by PCR out using primers designed to make them compatible for ligation-independent cloning (LIC) into the pTreTight vector. LIC is a process whereby so-called "sticky ends" on the insert correspond with matching sequences in the vector – allowing for the formation of covalent bonds and a new plasmid incorporating the GOI without the use of blunt ends or the need for ligases. This system allows for the control of insert directionality and has a high efficiency of GOI incorporation. The pTreTight-GOI vector can then be transduced by lipofection into the TetON clones from the previous round of cloning, and used to create inducible expression of the GOI under the regulation of the TetON promoter. Successful selection of clones that incorporate both the TetON insert as well as the GOI insert can be carried out through antibiotic selection with G418 and puromycin, since the cells with only the TetON vector contain a puromycin-susceptible trait that is spliced out with incorporation of the GOI in its stead.

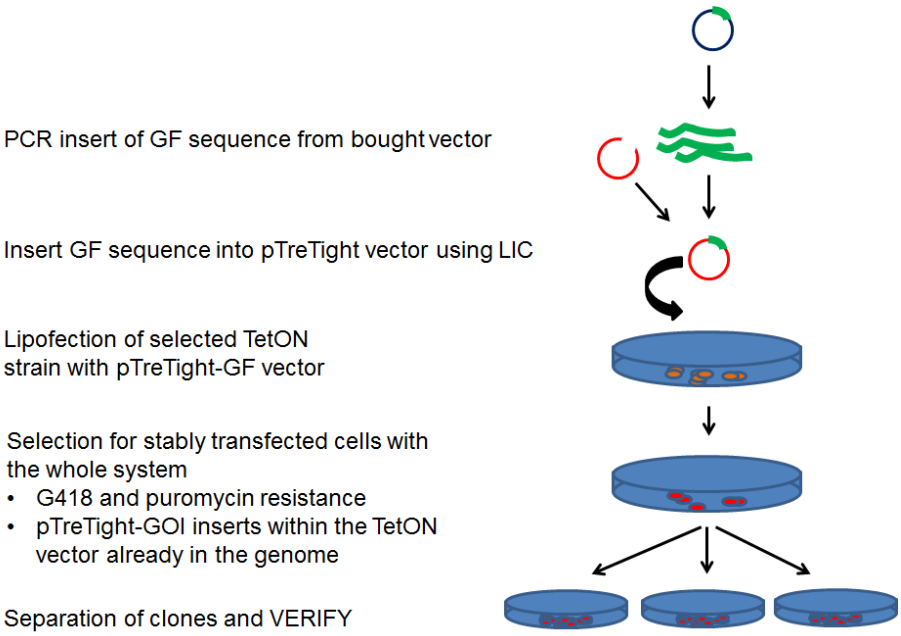



**Figure 5-2: Addition of the GOI to the TetON clone** selected for in Figure 5-1.

Once final TetON-GOI clones are separated and proliferated, Western blotting was needed to verify that the cells actually over-express (when treated with Dox) the GOI, i.e. VEGF or TGF-β. However, after much research, this was impossible to prove and the only remaining conclusion was that the cells did not over-express these growth factors. These cell populations had, after the first stage of cloning, been shown to express TetON-GFP, therefore there are essentially two options for where the transfection became misleading. The first hypothesis is that the TetON cells spontaneously became puromycin-resistant, without actually incorporating the gene. The second hypothesis is that, although transfection with the pTreTight gene led to incorporation of something into the TetON regulated loci, this was not actually a sequence that would successfully code for VEGF or TGF-β protein. Unfortunately, since DNA sequencing was never conducted on the pTreTight-GOI vectors, was not possible at that stage to determine which hypothesis was the correct one.

## 11882, 2496, and Saka Cell Line Performance *in Vivo*

When it was first decided to create cell lines that over-express vasculogenic, osteogenic factors the limitations of the project were immediately realized. hBMSCs change their gene expression over prolonged culture, even as short as five passages, *ex vivo*. On the other hand, cloning techniques generally have low efficiency, and therefore, once successfully transfected, the number of cell doublings in order to produce sufficient cells for a transplant ($2x10^6$ for even a small transplant as was studied in the thesis) would far exceed this limit. It was therefore decided to perform the first set of cloning in Oxford using three different human osteogenic cell lines (immortalized) in order to circumvent this problem.

There were 2 cell lines from ATCC (11882 and 2496) as well as one cell line obtained from a collaborator (David G Roodman, University of Pittsburgh, PA). CRL-11882 was a cell line created by transforming human bone marrow/stromal cells with HPV-16 E6/E7 and demonstrating fibroblast morphology. CRL-2496 was also created by transforming human bone marrow/stromal cells with HPV-16 E6/E7, but demonstrates epithelial morphology. All of these lines had been reported to support hematopoiesis *in vitro* [396, 397].

In parallel to attempts to transfect these cells using the TetON system, non-transfected cells were transplanted on Collograft (Zimmer, Warsaw, IN) product, an HA/TCP (reportedly 65% HA and 35% TCP) product frequently used in the Skeletal Biology Section (CSDB, NIDCR, NIH, DHHS). It is very important to know whether or not a cell line is capable of forming bone (and hematopoiesis) when placed in an *in vivo* transplantation, before attempting to modify it to improve its performance.

All cells were seeded at $2x10^6$ cells per transplant in NIH III nude mice and harvested after 12 weeks *in situ*. None of these "osteogenic, hematopoiesis-supportive" cell lines formed bone after 12 weeks – as demonstrated by their H&E histology below.

Therefore, it was decided that the second set of cloning would utilize a murine BMSC line that had spontaneously immortalized in the SBS lab and had been previously tested *in vivo* and shown to form bone and hematopoiesis on the same scaffold material.

11882 2496 Saka

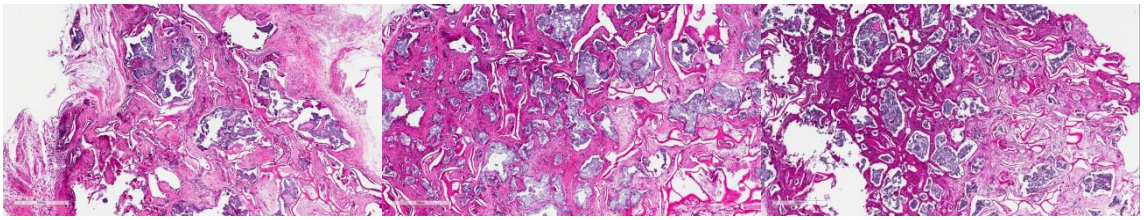


**Figure A5-3: H&E histology of osteogenic/hematopoiesis-supporting cell lines** on HA/TCP after 12 weeks. No bone or hematopoiesis is observed.

## Cloning Set 2 – NIH

After the first set of transfection of cells to over-express VEGF and TGF-β was unsuccessful, it was decided to attempt again, but using a different transfection system, and verifying every step of the way. An overview flowchart of the cloning process is shown below in Figure A5-4.

As before, GOI-containing plasmids were purchased and the GOIs were amplified into inserts using specifically designed primers. The ends of these inserts were then cut with restriction enzymes to create "sticky ends" and ligated into the "Gateway" entry vector pEntr3C (Invitrogen). The created plasmids were then sequenced to verify GOI insertion and integrity. Through LIC, the GOI was then moved into the PWPI-gateway vector that contains the lentiviral backbone. Again, GOI insertion and integrity was tested via DNA sequencing; even more importantly, the expression of protein from this plasmid was verified by transfecting the plasmid into 293 cells and demonstrating over-expression of VEGF and TGF-β. However, this set of cloning experiments unfortunately was terminated at this stage – with valid PWPI-VEGF and PWPI-TGF-β created.

This set of cloning experiments was stopped because research by another scientist in the CSDB (NIDCR, NIH, DHHS) into the viral transfection of cells revealed that the PWPI system presented unexpected challenges. When the spontaneously immortalized hBMSCs (discussed in the previous section of this appendix) were treated with lentivirus, most of the cells died. In contrast, when the same cells were treated with adenovirus the viability of the cells was maintained, but the cells showed no indication of actually accepting the GOI (test run was carried out using GFP vector). Thus, the decision was made to not spend any more effort on what would be a frustrating, time-consuming, and possibly fruitless endeavor.

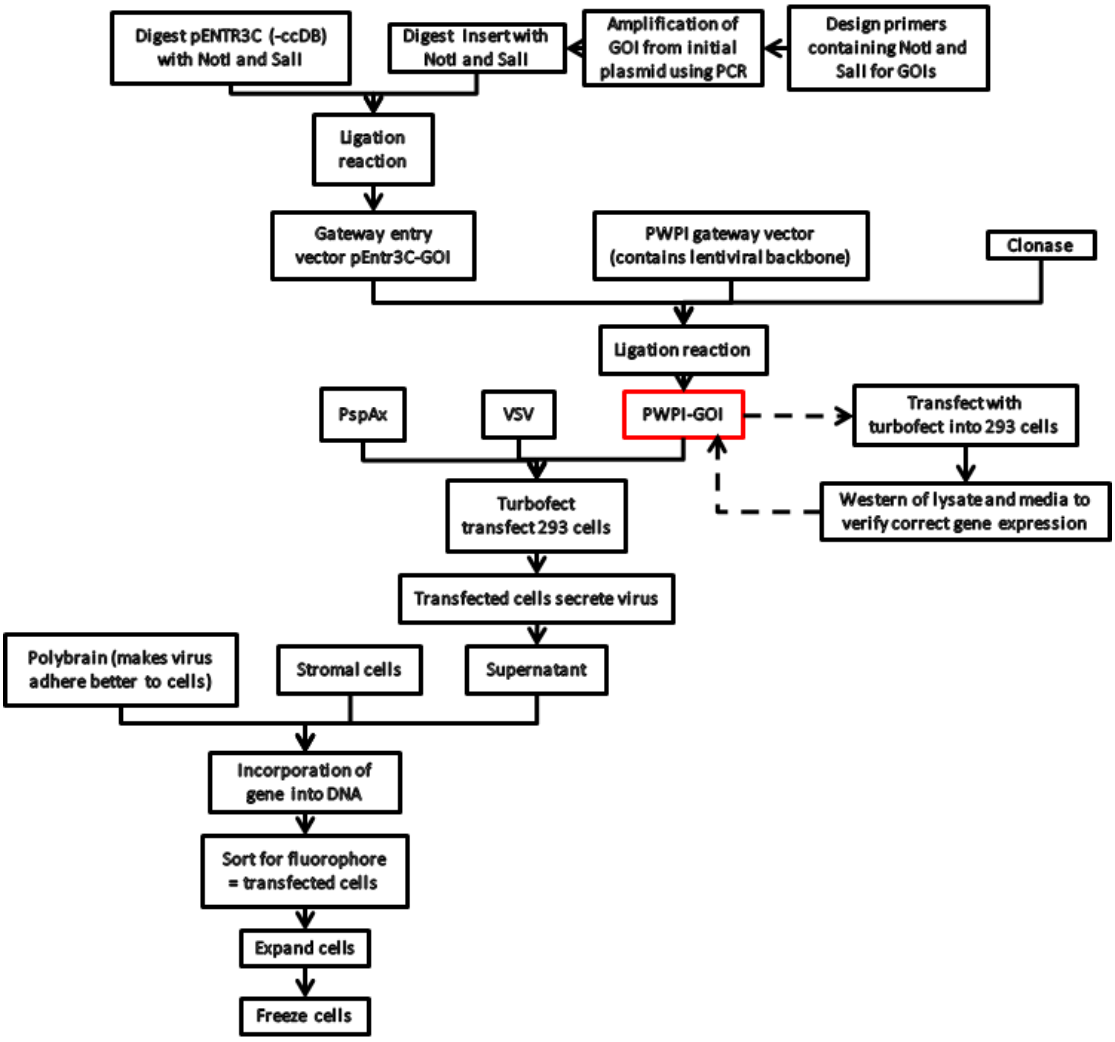



**Figure A5-4: Flowchart for the sequence of experimental actions taken to create virus-transfected cells. This set of cloning experiments progressed until the PWPI-GOI (red) product.**

# Appendix 6: Experiments Performed Post-Thesis Submission

## CFU-F count for FVB-N/J cells and C57BL/6J cells

### Justification

The current scientific opinions regarding skeletal stem cells and accurate measures of such cells and the isolation of such cells from mixed cultures were discussed in depth in the thesis. To summarize, all osteogenic stem cells are reflected in a CFU-F culture, however, not all CFU-Fs are skeletal stem cells. This is shown in Figure A6-1A. Figure A6-1B confirms that what we know is that BMSC cultures start out as only a tiny fraction of the whole bone marrow cell population, but is selected for by simple culture methods.

A) B)

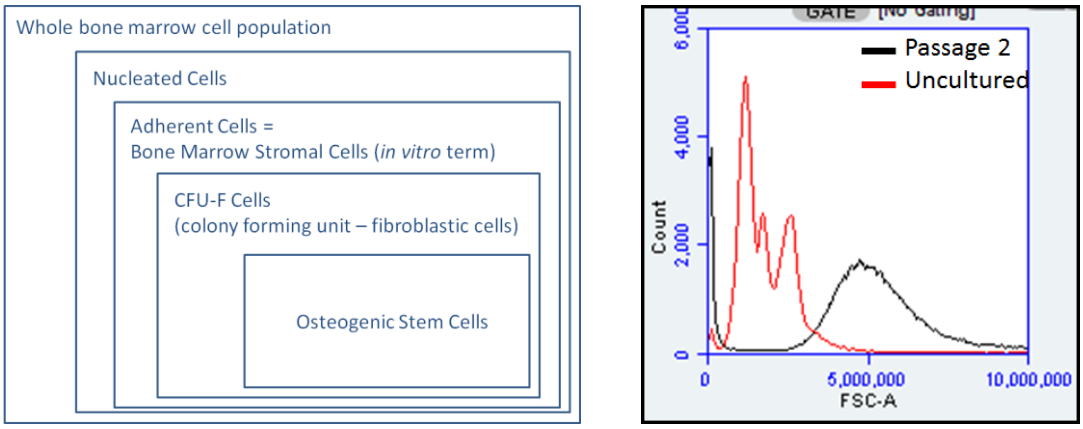



**Figure A6-1: A) Schematic of the cell populations within bone marrow.** Figure not to scale. **B) Comparison of murine cell populations** FACSed by size, directly after bone marrow flushing, and after 2 passages.

In the thesis (Chapter 3) it was shown that FVB-N/J derived mBMSCs can form bone and hematopoiesis in the ectopic transplant model in host FVB-N/J mice. This was found both when using individual FVB-N/J donors, and when pooling the bone marrow

cell population from 5 FVB-N/J mice. Interestingly, however, the pooled cell population not only produced less variable transplant performance, but also had better bone and hematopoiesis scores than the transplants seeded with cells from individual mice. This led us to see if we can correlate the performance of donor cells to their colony forming efficiency (CFE – whereby a CFU-F proliferates to form a colony) directly upon bone marrow harvesting. The CFE is an experiment that must be performed using the total primary cell population; therefore, it cannot be conducted retroactively for the FVB-N/J cell populations tested in the thesis. However, the experiment is being repeated with both CFE and *in vivo* ectopic ossicle model testing, in order to correlate the two.

Additionally, the experiment is being expanded to include a pooled BMSC population derived from C56BL/6J mice, in order to show variability between strains of mice, and test the syngeneic model on another strain of inbred mice.

## Materials and Methods

Colony forming efficiency (CFE) is not a technique presented in the thesis. Bone marrow cell populations were obtained from mice (protocol in Chapter 3), and then the bone marrow extract was broken down into a single cell suspension by pipetting up and down three times per 10 mL using a 16 gauge needle on a 10 mL syringe. Note that mice A-E and the pooled cells studied here are not the same mice as in the main body of the thesis. Additionally, the five mice from which the pooled cells were derived are different from the five mice whose cells were cultured individually. The solution was then passed through a 70μm sieve in order to remove any remaining cell clumps. 50μL cell solution was added to 5mL 3% acetic acid with methylene blue, mixed thoroughly and then nucleated cells were counted using a hemocytometer at 20x magnification. The volume for $4x10^6$ mononuclear cells was calculated, transferred to a 50 mL tube, made up to 24 mL using BMSC medium supplemented for a final concentration of 0.1mM β-mercaptoethanol. Four T25s received 6 mL each of this solution. The flasks were then

left undisturbed for 9 days. At 9 days the cells were rinsed with Hank's Buffered Saline Solution (HBSS – 5 mL for 2-3 minutes). This was then aspirated and 100% 5 mL methanol was added for 30 minutes. After this was aspirated, 5 mL methyl violet was added for 30 minutes, and rinsed with 2 full flask portions of distilled water under the faucet. Any remaining water was aspirated and then the samples were left to dry with the lid off on the bench. Colonies (the progeny of individual CFU-Fs) were counted under a dissection microscope in order to ensure that only colonies with greater than 30 BMSCs were counted, and not macrophage colonies or osteoclasts.

## Results

Each cell population was plated in quadruplicate with $1x10^6$ cells per T25. Thus, the units measured are colonies per $1x10^6$ nucleated cells.

A) B)

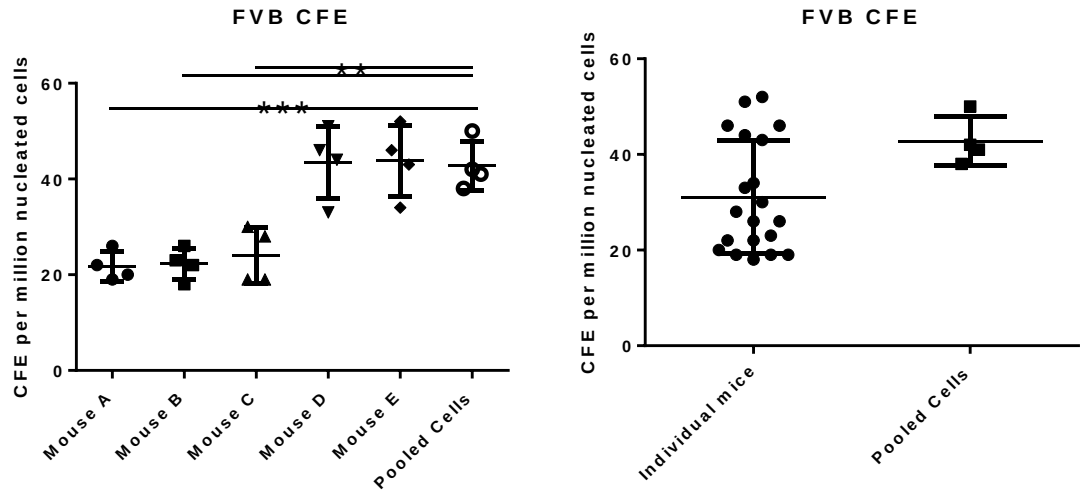



**Figure A6-2: A) Colony counts** from individual mouse donors (mice A-E) and from BMSC pooled from 5 FVB-N/J mice (additional mice, not mice A-E). B) The same results displayed as a simple average of the individual mice vs. pooled. ** = $p < 0.01$, *** = $p < 0.001$ n = 4 CFE assays performed per cell population

Figure A6-2 demonstrates the CFE variability present within the individual FVB-N/J populations. Although these mice are significantly inbred, and considered syngeneic for the purposes of this experiment, there is significant CFE variation from one donor

mouse to the next. The pooled cells have a CFE on par with the CFE from individual mice.

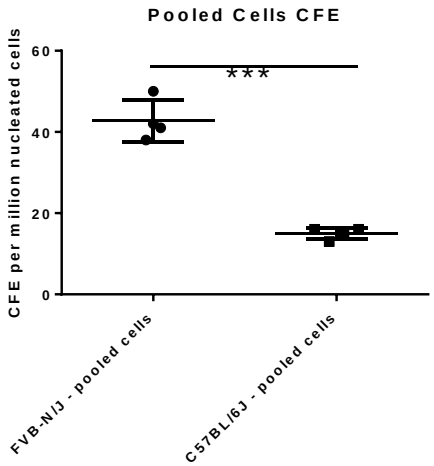



**Figure A6-3: Comparison of CFEs** of pooled BMSC populations derived from either FVB-N/J mice or C57BL/6J. *** = $p < 0.001$ n = 4 CFE assays performed per cell population

Figure A6-3 shows that bone marrow stromal cell populations derived from FVB-N/J mice have significantly higher colony forming efficiencies than BMSC populations derived from C57BL/6J mice.

## Discussion

The results from the colony forming efficiency assay have fairly straightforward interpretation, and support our *in vivo* findings. There was variability in performance between FVB transplants seeded with BMSCs from different donor mice – this is reflected in variability of CFE counts from individual donor mice. BMSCs pooled from several mice had higher bone and haematopoiesis scores than the average of the individual mice – this is reflected by the CFE count from pooled FVB mice being on par with the higher CFE count mice, and significantly different from the donor mice in the lower CFE group, instead of reflecting an average outcome.

Although *in vivo* syngeneic transplants using C56BL/6J BMSCs in host C56BL/6J have not been carried out, it is hypothesized that the CFE findings predict lower bone

and hematopoiesis scores for such transplants as compared to FVB-N/J syngeneic transplants.

### Conclusions and future work

This experiment is still in progress (at the time of thesis publication) in order to directly correlate CFE counts directly with performance in vivo in a syngeneic model. However, it is believed that CFE counts should directly correlate with performance and that these experiments demonstrate a number of key findings: that BMSC mediated bone and support of hematopoiesis formation in the syngeneic ectopic ossicle model is directly correlated to the number of CFU-Fs present in the transplanted cell population; that differences in CFE between strains of inbred mice is larger than the differences between mice within the strain, although inbred mice still produce donor variability; and that pooling cells somehow supports a higher level of CFE, and therefore better *in vivo* outcomes than BMSCs from individual donor mice.

## Osteogenic Assay for hBMSCs and a 50/50 mix of hBMSCs and HUVECs with various β-GP doses

### Justification

Previously in the thesis it was shown that at a 14 day timepoint, an equal combination of hBMSCs and HUVECs cultured in a standard osteogenic assay calcified significantly more than hBMSCs by themselves. However, the qPCR results taken from corresponding samples were confusing and did not necessarily correlate with osteogenic differentiation. This results from this assay, and some previous osteogenic assays conducted at the Skeletal Biology Section (CSDB, NIDCR, NIH, HHS), led to the observation that the so-called "osteogenic assay" probably should not be over-interpreted as an *in vitro* measure of osteogenesis, but rather simply of calcification.

Calcification could either be due to osteogenesis, or due to dystrophic calcification (dead and dying cells) or calcium phosphate (not hydroxyapatite) precipitation due to the action of alkaline phosphatase expressed by the cells. Based on previous literature, it was hypothesized that the driving force for dystrophic calcification in these experiments might be increasing levels of β-glycerophosphate (β-GP). We also hypothesized that it might be interesting to look at an earlier time point (7 days as contrasted to the 14 days previously examined) in order to see expression changes prior to significant amounts of calcification in the culture.

## Materials and Methods

Cells were plated at $0.1x10^6$ cells per well of a 6 well dish, either all hBMSCs or an even number mixture of hBMSCs and HUVECs. One hour after cell plating in 3 mL 5% serum-containing hBMSC medium, 330 μL of 10X concentrated osteogenic factor cocktail was added. Four different conditions for each cell type were tested (final concentrations shown).

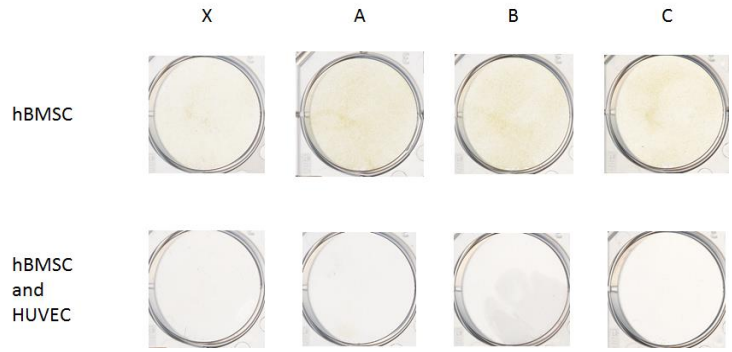
- Control: no osteogenic supplement added
- A: $1x10^{-3}$ M β-GP, $1x10^{-4}$ M AAP, and $1x10^{-8}$ M Dex
- B: $5x10^{-3}$ M β-GP, $1x10^{-4}$ M AAP, and $1x10^{-8}$ M Dex
- C: $1x10^{-2}$ M β-GP, $1x10^{-4}$ M AAP, and $1x10^{-8}$ M Dex

Media and factors were changed at 4 days, and additionally at 8 and 12 days for the 14 day experiment. At either 7 or 14 days the experiment was stopped and 3 wells from each plate were tested for Alizarin Red staining, to quantify calcification; while the other 3 were lysed for RNA extraction and qPCR testing. These methods are discussed in detail in Chapter 5 of the thesis. The housekeeper gene was GAPDH2.

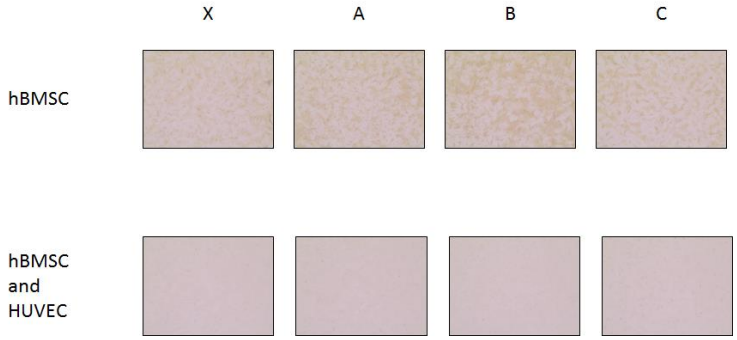
## Results

Samples did not calcify extensively over the course of this experiment as demonstrated by Figure A6-4. This was most likely due to the low density of the cells to start with, combined with the reduced serum conditions – and also because such assays are typically run for 21 or more days, with a minimum of 14 days. Thus, the experiment was less focused on calcification, and more on the gene expression that precedes the calcification (day 7) and through the early stages (day 14).

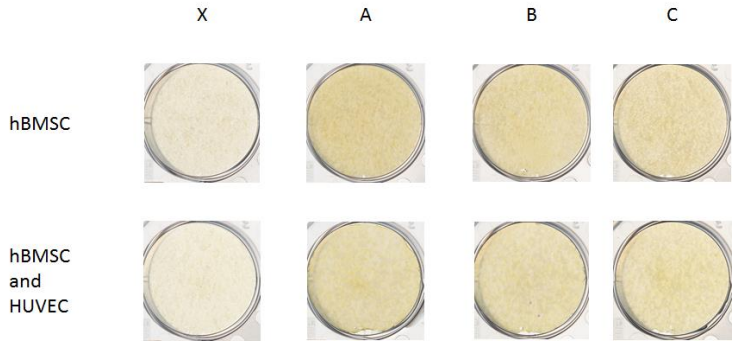
A)




B)




C)




D)

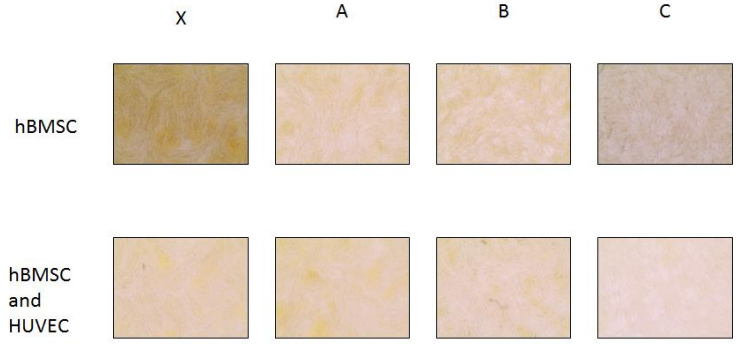



**Figure A6-4: A) scanned samples after 7 days B) 4x images of the samples after 7 days C) Scanned samples after 14 days D) 4x images of the samples after 14 days**. X = no osteogenic factors, A = osteogenic factors with 1mM β-GP, B = osteogenic factors with 5mM β-GP, C = osteogenic factors with 10mM β-GP

Figure A6-5 confirms, through quantification of Alizarin red staining, that there was very little calcification of any of the samples at day 7. This staining increased in all samples, including the controls, at day 14. A trend towards lower calcification at day 14 in samples with higher doses of β-GP was noted. At day 14 the two lower doses of β-GP had slightly higher calcification in the hBMSC samples than the mixed cell samples, but at 10mM β-GP, calcification levels (Alizarin red staining) were slightly higher in the mixed cell samples.

A) B)

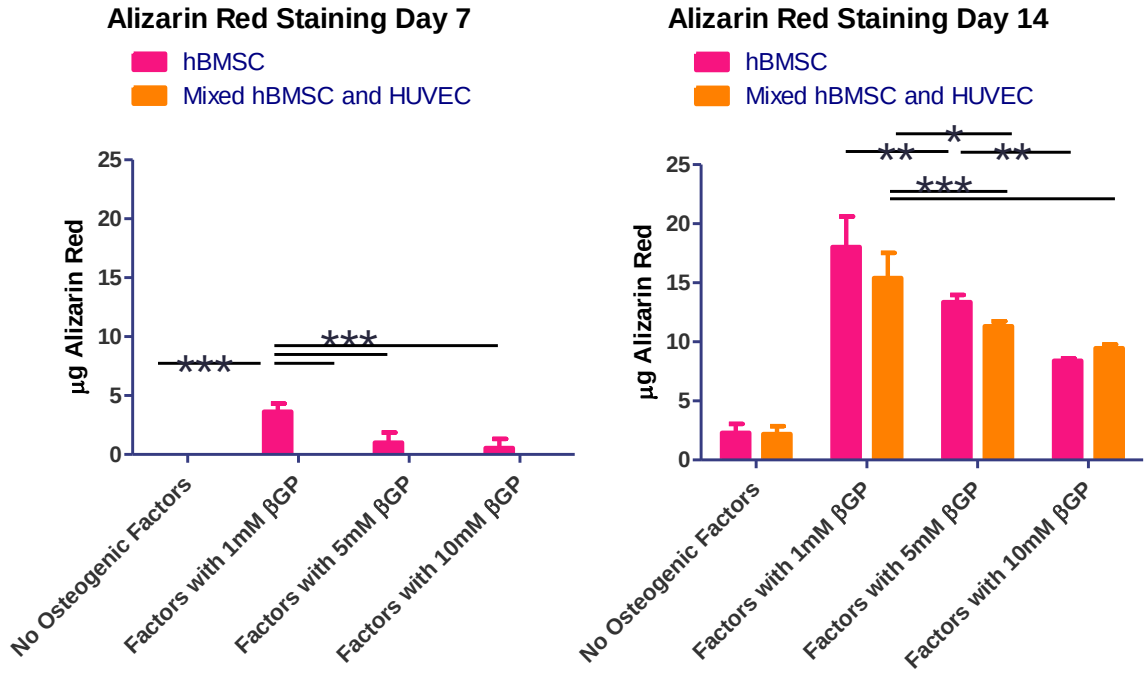



**Figure A6-5: Quantification of Alizarin red staining at A) day 7 and B) day 14**. At day 14, all samples were significantly higher ($p < 0.001$ than their relevant controls). * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ n = 3 wells for each condition used for alizarin red staining, each quantified in triplicate to minimize pipetting error.

Samples were analysed via qPCR for their RNA expression levels for alkaline phosphatase, bone sialoprotein, and osteocalcin. Figure A6-6 shows the gene expression response of either hBMSCs or HUVECs to various doses of β-GP at 7 and 14 days. Figure

A6-6 specifically examines the expression at 7 days as contrasted to 14 days, over both cell populations; with the highest dose of β-GP (only ALP results are shown as differentiation had not progressed enough for bone sialoprotein and osteocalcin levels to be significant).

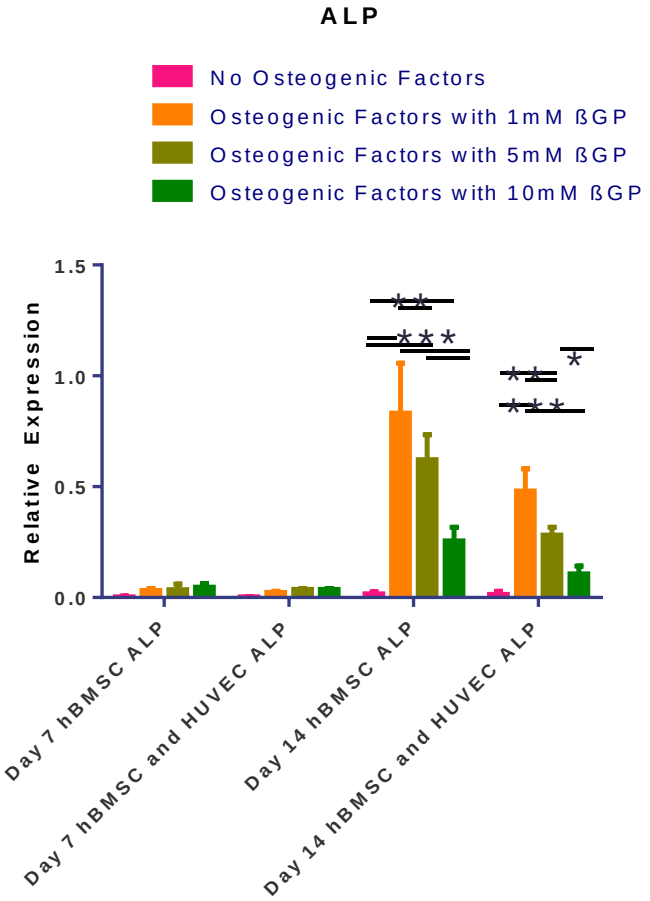



**Figure A6-6: Expression of alkaline phosphatase (ALP) at 7 and 14 days,** either in a pure hBMSC population, or a population containing HUVECs and hBMSCs. For 1mM and 5mM β-GP treated samples, differences between ALP expression were also significant at Day 14 between the hBMSCs and the hBMSC and HUVEC co-culture ($p < .001$ for each). * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ n = 3 wells for each condition used for qPCR, each qPCR analyzed in triplicate to minimize pipetting error.

Figure A6-6 shows that the alkaline phosphatase expression correlates with the alizarin red staining quantified in Figure A6-5. Although alkaline phosphatase expression was low at day 7, by day 14 it had increased significantly, particularly in samples with osteogenic factors, but lower doses of β-GP. Samples treated with higher doses of β-GP

exhibited decreased alizarin red staining, and also lower levels of expression of alkaline phosphatase, though still above the control of no osteogenic factors.

## Discussion

This experiment is an addition to the osteogenic assay performed in the course of this DPhil project, and presented in the main body of this thesis. That experiment found high levels of calcification, and, therefore, alizarin red staining, in samples that had a mixed cell population of hBMSCs and HUVECs. However, in that experiment, the qPCR results did not appear to correlate to the observed calcification. This incongruence led to the hypothesis that instead of true osteogenesis, the calcification observed actually represented dystrophic calcification. It was believed that this dystrophic calcification was driven by the β-GP in the osteogenic factor cocktail used. Thus, a new experiment was derived in order to test the effects of various doses of β-GP in the context of the osteogenic cocktail, with either hBMSCs alone (reference sample) or with our test cell population of half HUVECs and half hBMSCs (when seeded). Additionally, it was decided to evaluate alizarin red staining and osteogenic gene expression at days 7 and 14 in order to capture the early development of the cells under these conditions.

Unfortunately, due to technical difficulties, this experiment did not as such enhance our understanding of the results from the experiment in the main body of the thesis. The osteogenic assay is always difficult to carry out due to not only donor variability, but also the tendency of bone marrow stromal cells to contract away from monolayer culture to form a ball-like structure, when grown to a high enough density and when induced to produce large amounts of extracellular matrix. Thus, attempting to culture hBMSCs or a mixture of hBMSCs and other cells at confluence, without this balling phenomenon occurring for 7-21 days can be a finicky task. The assay procedure does not allow for trypsinization of cells to redistribute them at any point in the

experiment. Thus, due to time-constraints and after repeated failed attempts to successfully conduct the experiment with 10% serum-containing medium to mimic the previously published experiment, the experiment published here was conducted using medium containing 5% serum.

In the experiment detailed above, the alkaline phosphatase results reflect the results from the alizarin red staining. Samples treated with osteogenic factors produced higher levels of alkaline phosphatase expression and deposited higher amounts of calcium, than samples not treated with osteogenic factors. Of the samples treated with osteogenic factors, samples treated with higher doses of β-GP exhibited lower calcium deposition and inhibited alkaline phosphatase (ALP) expression as contrasted to those with lower doses of β-GP. This is the opposite of what had been the original expected outcome of this experiment. It had been hypothesized that high levels of β-GP would induce dystrophic calcification, leading to high levels of calcium deposition but from samples with very low ALP expression. In this instance it can only be postulated that this experiment is not an accurate reflection of what happened in the experiment presented in the main body of the thesis. It is proposed that, under low serum conditions, β-GP exerts a mild toxic effect on osteogenic cells, thereby inhibiting their ability to differentiate. Under higher serum-conditions, the hypothesis is that β-GP drives dystrophic calcification has not yet been disproved. In this experiment, the day 14 results demonstrate that despite this mild toxicity, and particularly at lower β-GP doses, osteogenesis does start to occur by day 14 in both the hBMSC and mixed cell populations treated with osteogenic factors – shown by ALP expression, which is a marker of early osteogenesis, but not by bone sialoprotein or osteocalcin expression (markers of late osteogenic development).

## Conclusions and future work

It is concluded that this experiment does not enhance our understanding of the *in vitro* distinction between dystrophic calcification and osteogenesis. However, it does expound on two key characteristics of the *in vitro* osteogenic assay. First, alizarin red staining alone should not be construed as osteogenesis in this type of assay – every time alizarin red staining is used, some other measure of osteogenesis should also be employed to ensure that this is true osteogenesis and not dystrophic calcification. For example, in this experiment, the low level of alizarin red staining observed was most likely a measure of osteogenesis, as it was supported by the qPCR ALP results – whereas in the previous experiment the alizarin red staining was exposed as dystrophic by the correspondingly low qPCR of osteogenic markers. Second, it is noted that these *in vitro* experiments are highly subject to the conditions in which they are carried out – cell density, and particularly, serum concentration of the medium being conditions that can have a large effect on the outcome of the experiment. Thus, one of the key observations from the thesis holds true – *in vivo* experiments remain the gold standard to measure the potential of cells to form true bone.