Optimal Biodegradable Scaffolds and Progenitor Cell For Effective Bone Regeneration

Ami R. Amini
aamini@student.uchc.edu

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Ami Rebecca Amini, PhD
University of Connecticut, [2014]

Bone tissue engineering has been proposed as a more effective and efficient alternative option for bone repair and regeneration. Here, we propose a two-pronged approach for enhance scaffold-guided bone regeneration. Second to developing optimized PLGA optimally-porous scaffolds, we will pre-vascularize our constructs in vitro in order to reduce vascularization time, and enhance bone formation in vivo. We will pre-vascularize our constructs by seeding and culturing them with a combination of two cell populations required for angiogenesis and osteogenesis: peripheral blood derived - endothelial progenitor cells (EPCs) and bone marrow derived -mesenchymal stem cells (MSCs). Previous studies have demonstrated EPCs and MSCs promote enhanced bone regeneration via the stimulation of neo-vascularization. We will systematically examine the combination of these two required cell populations and the optimally-porous PLGA scaffolds, and the resultant effects on healing critically sized segmental bone defects. We hypothesize that our pre-vascularized, optimally-porous PLGA scaffolds will substantially improve the performance of PLGA microsphere scaffolds by promoting angiogenesis, and significantly enhancing bone formation in vivo.
Optimal Biodegradable Scaffolds and Progenitor Cells for Effective Bone Regeneration

Ami Rebecca Amini
B.A., Stony Brook University, [2007]
D.M.D., University of Connecticut, [2014]

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Ami Rebecca Amini

[2014]
Doctor of Philosophy Dissertation

Optimal Biodegradable Scaffolds and Progenitor Cells for Effective Bone Regeneration

Presented by
Ami Rebecca Amini, B.A.

Major Advisor
__________________________________________
Cato T. Laurencin

Major Advisor
__________________________________________
Syam P. Nukavarapu

Associate Advisor
__________________________________________
Yusuf M. Khan

Associate Advisor
__________________________________________
Mina Mina

Associate Advisor
__________________________________________
Barbara E. Kream

University of Connecticut
[2014]
Life takes you to unexpected places,

Love brings you home.

I dedicate this thesis to my very special dad and mom, Ashley, Alexander, and Andrew for their unconditional love, support, encouragement, and always being proud of me.
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Figure 5-11. Representative cross-section MicroCT image of remodeled bone in the middle of the rabbit ulnar bone defect. U = ulnar bone, R = radius bone. Scale bar = 5 mm.

Figure 5-12. MicroCT analysis of ulnar bone defect 12 weeks post-implantation. Representative scout view images (i.e., radiographs), three-dimensional reconstructed images, and longitudinal section images are shown for each group (i.e., Acellular, MSC-seeded, EPC-seeded, and a 1:1 ratio of MSC:EPC seeded construct). Scout views were utilized to determine the region of interest (ROI) for bone mass and volume quantification. Scale bar = 5 millimeters.

Figure 5-13. (A) Bone Volume and (B) Mass with the ulnar bone defect. * indicates p < 0.05.
Figure 5-14. Representative histological images of Von Kossa–stained cross-sections (top two rows) and longitudinal sections (bottom two rows). Images were taken under low magnification to view the entire defect area in the section, and also under 4X magnification. Scale bar (low magnification) = 2 millimeters. Scale bar (4X magnification) = 500 µm.

Figure 5-15. Quantification of mineralized bone area formed within the rabbit ulnar defect. Three regions on Von Kossa stained sections were analyzed from 3 samples in each group under low magnification with a thresholding analysis tool from ImageJ software. ** indicates p < 0.05, ## indicates p < 0.001.

Figure 5-16. Representative histological images of Goldner’s Trichrome–stained longitudinal sections under 40X, 20X, 10X and 4X magnification. Scale bar for 40X images = 100 µm; Scale bar for 20X images = 250 µm; Scale bar for 10X images = 500 µm; Scale bar (4X magnification) = 500 µm.

Figure 5-17. Representative histological images of Goldner’s Trichrome–stained longitudinal sections under low magnification (Scale bar = 2 mm).

Figure 5-18. Quantification of collagen formation within the rabbit ulnar defect. Ten regions on Trichrome stained sections were analyzed for the intensity of collagen (green color) from 3 samples in each group under 20X magnification with a color (RBG) histogram analysis tool from ImageJ software (arbitrary scale of 0 to 255, with 255 representing the highest green intensity).

Figure 5-19. Quantification of vessels within the rabbit ulnar defect. Vessels were counted in five regions on Trichrome stained sections under 10X magnification for 3 samples in each group.
1. INTRODUCTION

Oral/maxillofacial and orthopaedic surgeons perform over two million bone replacement procedures annually, a figure lending bone as the second most transplanted tissue after blood (1, 2). Bone replacements/grafts are necessitated when patients experience bone loss due to high-energy trauma, tumor resection, revision surgery, developmental deformities, infection and non-union fractures, in addition to dental bone loss as a result of missing teeth and periodontal disease (3, 4). However, all currently available treatment options for bone repair or regeneration (i.e., autografts, allografts and synthetic materials) may present very serious risks. Although autografts are considered the “gold standard” and represent over half of bone substitutes, they are limited by the amount of available patient bone volume, and are also associated with donor site infection and morbidity (5-7). Allografts, isolated from cadavers, involve immunogenicity and disease transmission risks (8, 9). Also, synthetic materials (i.e., metals, plastics and ceramics) often require follow-up surgeries due to fatigue, fracture, and toxicity of the material (10). Thus, there is a warranted search for superior bone replacement methods to overcome the drawbacks of the current treatment options.

Bone tissue engineering (BTE) has been proposed as a more effective and efficient alternative option for bone repair and regeneration. BTE involves the combination of biodegradable and porous scaffolds, with or without the use of bone-forming cells and growth factors, to regenerate bone (11-14). The success of the scaffold-based bone regeneration approach critically depends on the effectiveness of the biodegradable scaffold used, as it serves as a temporary, mechanically-stable matrix for
cells to infiltrate and proliferate. Scaffold pore structure (i.e., pore size and interconnectedness) is an essential consideration in the development of scaffolds to ensure proper cell growth, migration and nutrient flow (15). Scaffolds currently designed with small pore sizes (i.e., approximately 100 µm) display in vitro and in vivo osteoblast survival and bone formation limited to the periphery, due decreased oxygen and nutrient diffusion throughout the scaffolds (16-22). On the other hand, studies have demonstrated scaffolds with large pore sizes (i.e., >400 µm) increase osteoblast proliferation and differentiation throughout the entire scaffold, due to enhanced mass transport of oxygen and nutrients and neo-vascularization (17, 23-26). However, scaffolds with larger pores are mechanically less stable than small-pored scaffolds, and are not mechanically compatible with human bone (27-30). Hence, sizing pores to achieve the necessary balance of cell growth and mechanical support is critical.

This work focuses on poly(85 lactide-co-15 glycolide) (PLGA) microsphere scaffolds. PLGA has been recognized for its biocompatibility, as well as its recent approval by the United States Food and Drug Administration (FDA) as a biodegradable material for certain biomedical devices (31, 32). For BTE applications, current PLGA scaffolds developed via a microsphere sintering technique have a unique advantage as they display mechanical properties in the range of human cancellous bone (31). However, the current PLGA microsphere scaffolds with small pore sizes (i.e. ~100 µm) fail to provide the prerequisites required for optimal bone regeneration (i.e. a stable oxygen and nutrient supply), limiting bone regeneration only to the scaffold surfaces (33, 34). We propose the generation of optimal PLGA microsphere scaffolds with optimally-sized pores to balance the positive and negative features of the previously mentioned pore
size ranges, and also allow for homogenous bone regeneration and neo-vascularization, while still retaining human bone-mechanical compatibility.

In addition to the fabrication of an effective BTE scaffold, establishing a sufficient vascular network in a timely manner is a crucial requirement for optimal bone tissue growth (35). This is clearly demonstrated in large BTE constructs that fail to support neo-vascularization throughout the construct, as seeded cells do not survive due to hypoxia and insufficient nutrition (36, 37). Thus, the rate and range of vascular growth determines the efficiency of new bone formation (38, 39). Although the objective of fabricating our PLGA optimally-porous scaffolds with increased interconnectivity and optimal engineering pore size is to enhance bone growth by improving neo-vascularization, this approach presents limitations. In situ neo-vascularization of tissue engineered bone is very slow to meet the nutritional requirements of osteoblasts, as it occurs over a course of several days to weeks for the center of the implanted constructs to become perfused (35).

Thus, we propose a two-pronged approach for enhance scaffold-guided bone regeneration. Second to developing optimized PLGA optimally-porous scaffolds, we will pre-vascularize our constructs in vitro in order to reduce vascularization time, and enhance bone formation in vivo. We will pre-vascularize our constructs by seeding and culturing them with a combination of two cell populations required for angiogenesis and osteogenesis: peripheral blood derived -endothelial progenitor cells (EPCs) and bone marrow derived -mesenchymal stem cells (MSCs) (40-48). Previous studies have demonstrated EPCs and MSCs promote enhanced bone regeneration via the stimulation of neo-vascularization (47, 49, 50). We will systematically examine of the combination
of these two required cell populations and the optimally-porous PLGA scaffolds, and the resultant effects on healing critically sized segmental bone defects. We hypothesize that our pre-vascularized, optimally-porous PLGA scaffolds will substantially improve the performance of PLGA microsphere scaffolds by promoting angiogenesis, and significantly enhancing bone formation \textit{in vivo}. Upon the completion of the proposed specific aims, we will have addressed a significant challenge in the field of scaffold-based BTE and its clinical applicability towards bone defect repair and regeneration in oral/maxillofacial and orthopaedic surgery.
2. BACKGROUND

Bone grafts are utilized in a wide array of clinical settings to augment bone repair and regeneration. Bone defect repair via tissue engineering approach is perceived as a better approach as the repair process may leave with the patient own tissue by the time the regeneration is complete (11, 51, 52). Currently, the United States, as well as other countries worldwide, are experiencing an exceedingly high demand for functional bone grafts, as the statistics have risen above half a million recipient patients and costing over $2.5 billion annually in the United States. This figure not only doubles on a global basis, but is also expected to double by 2020 due to a variety of factors, including the growing baby boomer population and increased life expectancy (53).

Extensive studies have reported on considerable shortcomings, limitations and complications of current clinical treatments for bone repair and regeneration, which include autologous and allogeneic transplantations by using autografts and allografts (Table 2-1) (5, 7, 53-57). To date, autografts serve as the gold standard for bone grafts as they are histocompatible and non-immunogenic, and offer all the imperative properties required of a bone graft material. Specifically, autografts possess the essential components to achieve osteoinduction (i.e., bone morphogenetic proteins (BMPs) and other growth factors), osteogenesis (i.e., osteoprogenitor cells) and osteoconduction (i.e., 3D and porous matrix). However, autografts involve harvesting bone from the patient’s iliac crest, and thus, requires a second operation at the site of tissue harvest (58). Autologous bone transplants are very expensive procedures, and may result in significant donor site injury and morbidity, deformity, scarring, as well as surgical risks, including
bleeding, inflammation, infection and chronic pain (6, 59, 60). Autografts, further, may be a null treatment option in cases where the defect site requires larger volumes of bone than is feasible or available. Allografts represent the second most common bone grafting technique and involves transplanting donor bone tissue, often from a cadaver. Allogeneic bone is also likely histocompatible, and is available in various forms, including demineralized bone matrix (DBM), morcellised and cancellous chips, cortico-cancellous and cortical grafts, and osteochondral and whole-bone segments, depending on the host site requirements. In comparison to autografts, allografts carry risk of immunoreactions and transmission of infections, and reduced osteoinductive properties and no cellular component, since donor grafts are devitalized via irradiation or freeze-drying processing (8-10). Although less than autografts, allogenic grafts come with substantial cost issues. Further, the bone grafting market is experiencing an obvious unmet supply and demand as there is currently a shortage in allograft bone graft material (61). Other commonly used bone repair techniques may involve distraction osteogenesis, bone cement fillers and bone morphogenic proteins. Although the previously mentioned clinical interventions have been shown to improve repair of bone, none possess all the ideal characteristics: high osteoinductive and angiogenic potentials, biological safety, low patient morbidity, no size restrictions, ready access to surgeons, long shelf life, and reasonable cost.

The field of bone tissue engineering (BTE) was initiated nearly three decades ago. As seen in Figure 2-1, interest and progress in the BTE field has seen tremendous growth over the years, with and exponentially increasing number of studies and reviews published on the PubMed database since the mid-1980s. The field of BTE is aimed to create an alternative treatment option that would ideally eliminate the previously
described issues of current clinically used treatments (i.e., donor site morbidity, limited availability, immune rejection and pathogen transfer) (62). BTE requires the collaborative efforts of scientists, engineers and surgeons alike in order to achieve this ultimate goal of creating bone grafts that enhance bone repair and regeneration. The classic BTE paradigm highlights several key players; (i) a biocompatible scaffold that closely mimics the natural bone extracellular matrix niche, (ii) osteogenic cells to lay down the bone tissue matrix, (iii) morphogenic signals help direct the cells to the phenotypically desirable type, and (iv) sufficient vascularization to meet the growing tissue nutrient supply and clearance needs. Specifically, as seen in Figure 2-2, upon implantation, the construct may influence the host by releasing osteogenic and/or

![Figure 2-1](image.png)

Figure 2-1. (A) Published articles on bone tissue engineering since mid-1980s on PubMed. (B) Break-down of the articles published in 2011 according to bone tissue engineering focus (i.e., biomolecules, cells, matrices, and other, including vascularization approaches and bioreactors).
Table 2-1. Bone graft options for bone repair.

<table>
<thead>
<tr>
<th>Bone Graft</th>
<th>Structural Strength</th>
<th>Osteo-conduction</th>
<th>Osteo-induction</th>
<th>Osteo-genesis</th>
<th>Disadvantages</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Autograft</td>
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</tbody>
</table>
| Cancellous          | No                  | +++              | +++             | +++           | - considered gold standard for repairs of bone defects  
| Cortical            | +++                 | ++               | ++              | ++            | - contains osteoprogenitor cells capable of synthesizing new bone, and a structural matrix that acts as a scaffold  
|                     |                     |                  |                 |               | - limited supply  
|                     |                     |                  |                 |               | - donor-site pain, infection, morbidity can be as high as 25%  
|                     |                     |                  |                 |               | - required secondary surgery                                                                                                                  |
|                      |                     |                  |                 |               | Allograft                                                                                                                                 |
| Cancellous          | No                  | ++               | +               | No            | - immunogenic response by the host to the foreign tissue of the graft  
| Cortical            | +++                 | +                | +               | No            | - potential for disease transmission  
| De-mineralized Bone Matrix (DBM) | No | + | + | No | - prepared from cadaveric human bone  
|                      |                     |                  |                 |               | - contains noncollagenous proteins: type 1 collagen (provides the osteoconductive scaffold for osseous in-growth), and osteoinductive growth factors (BMPs, fibroblast growth factor, insulin-like growth factor, platelet-derived growth factor, and TGF-β)  
|                      |                     |                  |                 |               | - available in several forms, including freeze-dried powder, granules, gel, putty, and strips                                                                 |
|                      |                     |                  |                 |               | Bone graft substitutes                                                                                                                        |
| Coralline HA        | +                   | ++               | No              | No            | - FDA approved in 1992  
|                     |                     |                  |                 |               | - produced from marine coral exoskeleton that have pore structures resembling cancellous bone  
|                     |                     |                  |                 |               | - effective for managing metaphyseal defects  
|                     |                     |                  |                 |               | - not highly resorbable: blocks of implanted coralline HA remain for 10 years                                                                 |
| Collagen-based matrices | No | + | No | No | - approved by FDA in 1991  
- xenografts consisting of sponge-like strips of bovine collagen combined with hydroxyapatite  
- Collagraft (Collagen / HA / Tri-calcium phosphate, Zimmer), Healos (Collagen / HA, Depuy) |
| Calcium phosphate | +++ | ++ | No | No | - capable of osteoconduction and osseointegration  
- injectable calcium paste has 4 to 10 times the compressive strength of cancellous bone  
- calcium phosphate cement has the highest mechanical compression strength of any of the osteoconductive bone-graft substitutes  
- 95% of calcium phosphate is resorbed in 26-86 weeks |
| Calcium sulfate | No | + | + | + | - approved by FDA in 1996  
- calcium sulfate resorbs in 4-12 weeks, making it the quickest of any of the osteoconductive products currently available  
- wound drainage occasionally is noted and is hypothesized to be the result of the osmotic effect of the calcium sulfate  
- osteoconductive void filler  
- available from numerous companies (Osteoset, Calceon 6, Bone Blast, etc.) |
| Bioglass | + | ++ | ++ | ++ | - mimics mineral composition in bone |
| Biodegradable Polymers (i.e., PLGA) | + | ++ | No | + | - tunable porosity and mechanical strength depending on form  
- various polymers (PLGA, PLLA) release acidic byproducts  
- abundant supply  
- no risk of disease transmission |
vasculogenic growth factors (i.e., growth factor-releasing scaffold, scaffold with growth factor analogs, or seeded with platelet-enriched plasma), or by housing cells that are genetically engineered to or naturally release growth factors. In turn, accelerated cell homing, vascularization and bone regeneration of the defect site results. Although much progress has been made, there still are many crucial hurdles yet to be cleared on the way to BTE becoming a true clinical reality. The following is a review of critical consideration, advances and obstacles for functional BTE.

Figure 2-2. Schematic illustration of bone tissue engineering paradigm. Factors from the implanted graft at the defect site that influence the host response may include growth factors (or their analogs, or from platelet-enriched plasma), and cells (genetically- modified to release factors, or naturally produce factors). In response, cell homing and enhanced vascularization and bone regeneration will occur.
2.1. Fundamentals of Bone And Developmental Biology

Bone tissue engineering (BTE) is based on the understanding of bone structure, mechanics and tissue formation as it aims to induce new functional bone tissues. In other words, in order to successfully regenerate or repair bone, knowledge of the bone biology and its development is quite essential.

Bone possesses the ability to perform a wide array functions, and respond to a variety of metabolic, physical and endocrine stimuli. Bones represent (1) the foundation for our bodily locomotion, (2) provide load-bearing capacity to our skeleton and protection to our internal organs, (3) house the biological elements required for hematopoiesis, (4) trap for dangerous minerals (i.e., lead), as well as (5) maintain the homeostasis of key electrolytes via calcium and phosphate ion storage. In addition, it is engaged in a constant cycle of resorption and renewal, undergoing continual chemical exchange and structural remodeling due to both internal mediators and external mechanical demands (11). Bone has been previously, and most appropriately referred to as the ultimate smart material for its scar-less regenerative capacity. Functional bone tissue engineering requires that the newly restored bone to be fully integrated with the neighboring host bone, and importantly, possess the ability to perform the previously mentioned functions of native bone.

Bone is a highly dynamic and diverse tissue both, structurally and functionally. Macroscopic structure and mechanical properties of the various two hundred plus bones
in the human skeletal system are largely influenced by distinct loading conditions. Skeletal structures range from being long (i.e., tibia, ulnar, etc.), short (i.e., phalanges, etc.), flat (i.e., skull, sternum, etc.) and irregular (i.e., pelvic, vertebrae, etc.). Bone functions range from locomotion, to vital organ protection. Bone tissue may also either take on a compact (i.e., cortical bone) or trabecular (i.e., cancellous bone) pattern arrangement, ranging in mechanical strength and modulus. Despite these complex features and forms, it has a relative simplicity in terms of its microscopic, hierarchical architecture. Specifically, bone extracellular matrix (ECM) is comprised of both a non-mineralized organic component (predominantly type-1 collagen) and a mineralized inorganic component (composed of 4 nm thick plate-like carbonated apatite mineralites). The nano-composite structure (tough and flexible collagen fibers reinforced by hydroxyapatite crystals) is integral to the requisite compressive strength and high fracture toughness of bone (63).

2.1.1. Bone Development

Bone formation occurs via two very distinct pathways (intramembranous and endochondral). In either case, mesenchymal cellular condensation first occurs and serves as a template for the subsequent bone formation. Intramembranous bone formation involves mesenchymal progenitor cells differentiating directly into osteoblasts, and the subsequent development of parts of the mandible and clavicle, and many cranial bones. Most bones in the body (i.e., all long bones and vertebrae), however, are formed through endochondral bone formation. This process involves mesenchymal progenitor cells first
differentiating into chondrocytes, which are responsible for depositing a cartilaginous template that is later, mineralized and replaced by bone.

Although there are distinct differences in the bone composition and structure formed via endochondral and intramembranous ossification, several molecular regulators are shared (64, 65). For instance, several key molecules including Indian Hedgehog (Ihh), parathyroid hormone related peptide (PTHrP), bone morphogenetic proteins (BMPs), vascular endothelial growth factor (VEGF) and fibroblastic growth factors (FGFs) are critical regulators in both processes (66). In endochondral ossification, BMPs are responsible for the initiation of mesenchymal condensations, and Ihh and PTHrP form a critical feedback loop that mediate the balance between chondrocyte proliferation and hypertrophy and regulate the thickness of the growth plate. Likewise, during intramembranous bone formation, these key players are required to induce uncommitted mesenchymal progenitor cells along the osteogenic pathway as pre-osteoblasts, which co-express chondrocytic and osteoblastic markers simultaneously. Furthermore, in both processes, bone remodeling is required for the maintenance of all normal healthy bone, which involves a balance between osteoclastic bone resorption and osteoblastic bone formation (67).

### 2.1.2. Bone Defect Repair

Interestingly, upon fracture, bone is repaired by a process that recapitulates many of the events of both intramembranous and endochondral bone formation, and uniquely heals without the formation of scar tissue (Figure 2-3) (68, 69). Initially, hematoma formation is accompanied by an inflammatory response, and the recruitment of many of
the signaling molecules involved in the regulation of new bone formation (i.e., ILs, TNF-α, FGFs, BMPs, PDGF, VEGF, etc.). At the cortex and periosteum, intramembranous

![Diagram of bone defect repair steps](image)

**Figure 2-3.** Schematic illustration of steps involved in bone defect repair.

bone formation immediately occurs. The external soft tissues stabilize the fracture by the formation of a callus, which subsequently undergoes chondrogenesis, and then a process highly similar to endochondral ossification. More specifically, after the callus forms, chondrocyte proliferation decreases, as they begin to mature (i.e., hypertrophy) and calcify the matrix. In-growing blood vessels carry in chondroclasts, which are responsible for resorbing the calcified cartilage, and osteoblastic progenitors, which begin the process of new bone formation. The mechanical continuity of the cortex is achieved via subsequent remodeling of the newly formed bone.
The question remains, what is the optimal method for bone regeneration? Should BTE focus more on bone development processes or bone defect repair? In my opinion, BTE should not exclusively focus on one or the other, but instead both. In situations requiring bone regeneration, the initial events always involve hematoma formation and an early inflammatory response, which is largely responsible for the recruitment of host cells and release of critical signaling molecules. From there, emulation of some aspects of normal bone tissue development and remodeling may hold the key to the future success of BTE. Seminal developmental biology principles that may help the future success of BTE include:

1. the use of pluri- or multi-potent stem cells;
2. the identification of critical genes, growth factors, and signal transduction cascades that mediate bone formation;
3. the physical process of bone formation;
4. complex interactions between epithelium and mesenchyme within the underlying connective tissue;
5. the understanding of mesenchyme encoding tissue-specific patterns;
6. the understanding that normal tissue healing involves progressive remodeling and restructuring of pre-existing tissue structures,
7. the importance of the tissue microenvironment’s physical properties (i.e., “mechanotherapy”) and
8. angiogenesis and neo-vascularization of the newly formed bone tissue.

Incorporation of developmental biology insights will critically impact future
tissue engineering approaches. For instance, inclusion of appropriate extracellular matrix molecules or adhesive ligands that target stem cells mediating earlier stages of tissue remodeling and regeneration (70). Also, for the promotion of angiogenesis, development of scaffolds that incorporate growth factors and possess the necessary porosity for vascular ingrowth (16). Further, engineering micro- and nano-meter featured surface topography of these scaffolds is critical for directing cellular adhesion, spreading, and proliferation. On a broader scale, for successful BTE, it is critical to develop a scaffold that is inspired by the natural processes of developmental biology and promotes tissue remodeling, and not just simply supports final tissue form and function.

2.2. Recent Advances in Bone Tissue Engineering (BTE)

Although bone is a highly vascularized tissue and has the ability to regenerate, beyond a critical point, clinical intervention measures are required. It is the hope that bone tissue engineering will be the future treatment of choice, as it will likely eliminate many of the pitfalls and concerns of current treatments. Here, I discuss the status and key issues for BTE components (i.e., scaffolds, cells and vascularization).

2.2.1. Biodegradable Scaffolds

2.2.1.1. Scaffold Mechanical Integrity
A key feature of BTE scaffolds is to provide temporary mechanical integrity at the defect site, until the bone tissue is repaired or regenerated, and normal biomechanical function is restored. It is established that in order for the bone tissue engineering scaffold to be “functional” immediately upon implantation, its biomechanical properties must match the physical demand of the healthy surrounding bone (71). In addition, mechanical strength of the scaffold affects the mechanotransduction of the adherent bone cells on the scaffold, which plays a critical role in the bone repair and remodeling processes. It has been proposed that, generally, the structural biomechanics of the BTE scaffold is related to the osteoconductive properties of the scaffold, while mechanotransduction is related to its potential osteoinductive properties (72). Biomechanical stimuli on cells due to the scaffold deformation largely influences osteoinduction (i.e., bone ingrowth from the host). Therefore, as suggested by Sikavitsas et al., a mechanotransduction strategy may be used to control the function of bone cells in vivo by designing a scaffold with mechanical properties that allow ‘osteoinductive fluid flow’ in the scaffold. By combining 3D imaging and numerical simulation of scaffold physical properties, it was verified that a threshold permeability of $\sim 3 \times 10^{-11} \text{ m}^2$ was necessary for inducing vascularization and mineralization in the bone graft (73, 74).

The BTE biomechanical paradigm has been well described in a step-wise fashion, where each step holds the mechanical aspects of the scaffold central to insure the safety of the surgical procedure using a BTE scaffold (Figure 2-4) (72). The first step, which involves the bone mechanical properties and loading conditions, are analogous to the primary fixation of the scaffold. At this point, the BTE scaffold should not induce a stress-shielding effect, resulting in peri-scaffold bone resorption as seen in the metallic
joint implants. Also, the elastic property of the BTE scaffold should not exceed that of

![Figure 2-4. Illustration of a three-step biomechanical paradigm in BTE. In the first step, upon implantation, it is critical that the mechanical properties of the BTE scaffold closely match that of the surrounding host bone tissue and loading conditions to reduce the stress-shielding effect. The second step involves interface biomechanics, and should allow for interface scaffold-bone mechanotransduction for enhanced osteointegration of the scaffold. Lastly, as the scaffold degrades, ingrowing bone tissue will begin to support the mechanical load of BTE scaffold. Adapted from Pioletti et al. (72).](image)

bone in order to maintain a proper mechanical stimulation on the peri-scaffold bone, which is driven by the loading conditions. The second step involves interface biomechanics, and may be identified as the secondary fixation. Here, the mechanical properties of the BTE scaffold may be adapted to generate interface scaffold-bone mechanotransduction, which has been shown to influence tissue differentiation and osteointegration of the scaffold (75). For the third step, which may be termed ‘final fixation,’ involves scaffold evolution, and the ingrowing bone offering support to the mechanical load as the BTE scaffold degrades. Thus, each step revolves around mechanical aspects, which induce a biological reaction in and around the BTE scaffold via mechanotransduction. It has been suggested that the separations between these steps may be utilized as an engineering approach in the mechanical design of bone scaffolds.
Ideally, if mechanical considerations can be used to confer osteoinductivity to a BTE scaffold, the dependency of using osteogenic factors and bioreactors may be reduced, and an off-the-shelf product could be obtained (76).

Mechanical properties of human bone vary tremendously according to location and function (i.e., load or non-load bearing). Again, the restorative scaffold’s mechanical properties should be modulated or tailored to match the demands of that at the defect site, in order to decrease or avoid complications, such as stress shielding, implant-related osteopenia and subsequent re-fracture (77).

**2.2.1.2. Scaffold Porosity**

Micro-porosity is a critical element for the scaffold’s osteoconductive properties, and the resultant bone tissue ingrowth and vascularization. Scaffold pore structure (i.e., pore size, volume and interconnectedness) is an essential consideration to ensure proper cell growth, cell migration, nutrient flow, vascularization, and better spatial organization for cell growth and ECM production (15, 19). Although there is still some ambiguity surrounding the optimal porosity and pore size for a 3D bone scaffold, studies suggest that scaffolds currently designed with small pore sizes (i.e., < 200 µm) display *in vitro* and *in vivo* osteoblast survival and bone formation limited to the periphery, due decreased oxygen and nutrient diffusion throughout the scaffolds (16). On the other hand, scaffolds with a mean pore size above 200 µm display increased osteoblast proliferation and differentiation throughout the entire scaffold, due to enhanced neo-vascularization and mass transport of oxygen and nutrients (17, 23-25). For instance, Tsuruga *et al.* demonstrated enhanced bone formation, as well as alkaline phosphase and osteocalcin
levels in constructs with mean pore sizes of 300 to 400 µm four weeks post-implantation in a rat subcutaneous mouse model (17). In addition, Klenke et al. demonstrated a positive relationship between the scaffold’s mean pore size and vascular invasion, volume of newly-formed bone, and bone volume density. After 4 weeks, implantation of scaffolds with pore sizes 200-300 µm resulted in significantly enhanced vascular and bone formation than that with pore sizes less than 200 µm in a mouse critical-size cranial defect model (78).

A combination of various factors may attribute to the observed enhanced performance of scaffolds with pore sizes greater than 200 µm. Scaffolds with larger pores result in decreased cell aggregations developed along the scaffold’s periphery. Specifically, Murphy et al. observed that scaffolds with pore sizes greater than 300 µm demonstrate improved cell migration, which overcome the scaffold-surface cell trapping phenomenon in vitro (79). Instead, scaffolds with larger pore sizes allow for homogenous cell proliferation through the construct, of which is not limited to the scaffold periphery. Minimizing the cell sheet formation at the scaffold’s periphery may also allow for enhanced diffusion of oxygen and nutrient. Hypoxia, which defines the lack of oxygen, is well associated with osteoblastic cell death. Therefore, it is critical to fabricate scaffolds with proper pore sizes to allow for oxygen diffusion throughout the scaffold. Together, these factors allow for enhanced cell survival throughout the scaffold, and not central necrosis that is often observed in scaffolds with small mean pore sizes.

The cruciality of increasing pore size of scaffolds is well recognized, and a variety of scaffold fabrication techniques have been proposed to achieve scaffolds with increased
pore sizes. Porogen leaching was used in combination with several traditional scaffold fabrication techniques, such as gas foaming (80, 81), freeze drying (82), and phase separation (83) to fabricate highly porous scaffolds. However, as porosity and mean pore sizes increase, mechanical strength is scarified; determination of a balance between mechanical strength and porosity is crucial.

2.2.2. Cellular Approaches

Though there remains an unresolved debate on the most effective cell type for clinical bone regeneration, it has been established that cellular-based bone regeneration approach is indeed effective. Cellular-based approaches in BTE primarily target the early stages of bone repair when the recruitment of skeletal progenitors may be impaired. Proposed mechanisms by which implanted cells enhance bone regeneration in BTE involve (1) early release of key osteogenic and vasculogenic molecules and growth factors, and (2) formation of a template to recruit host osteogenic and vasculogenic cells, and (3) actively laying down bone matrix and vascularizing the bone construct.

The major challenge in making these cellular therapies more efficient is the identification of the cell sources that can be implanted to the bone defect site, and will differentiate into osteoblasts and form neo-vasculature (84, 85). Thus far, studies have investigated several cell types including mesenchymal stem cells (MSCs), embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), adipose derived stem cells (ADSCs) and stem cells from human exfoliated deciduous teeth (SHED) for their abilities to promote bone repair and regeneration. This variety of possible candidates for cell transplantation can be explained by the finding that cells involved in the reconstruction of osseous tissue undergo a progression from undifferentiated progenitors to
biosynthetically mature cells; therefore therapeutic strategies can approach supporting the healing process at different stages of bone tissue development (86). For successful clinical application in the regeneration of bone, the properties of choice include isolation and expansion efficiency, expression and stability of osteogenic markers, “bona fide” bone tissue formation, and long-term safety (i.e., immunorejection, graft-versus-host

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Source</th>
<th>Clinical Use</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Embryonic Stem Cells</strong></td>
<td>Embryonic Bodies (EBs)</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Induced Pluripotent Stem Cells (iPSCs)</strong></td>
<td>Any cell type that could be induced to become osteoblasts</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Adult Stem Cells</strong></td>
<td>- Bone marrow</td>
<td>• Segmental defects of long bones</td>
</tr>
<tr>
<td></td>
<td>- Adipose tissue</td>
<td>• Large bone diaphysis defects</td>
</tr>
<tr>
<td></td>
<td>- Peripheral Blood</td>
<td>• Maxillary sinus augmentation</td>
</tr>
<tr>
<td></td>
<td>- Teeth (pulp, exfoliated teeth)</td>
<td>• Posterior spinal fusion</td>
</tr>
<tr>
<td></td>
<td>- Cord blood</td>
<td>• Bone tumor resection</td>
</tr>
<tr>
<td></td>
<td>- Amniotic Fluid</td>
<td>• Large calvarial defect</td>
</tr>
<tr>
<td></td>
<td>- Stem Cells derived from ESCs and iPSCs</td>
<td>• Osteonecrotic Femoral Heads</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Hip osteonecrosis</td>
</tr>
<tr>
<td><strong>Genetically Modified Cells</strong></td>
<td>Any cell type that could be genetically modified</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Autologous Cells and Growth Factor Cocktail</strong></td>
<td>Platelet Rich Plasma Bone marrow Aspirate</td>
<td>• Necrosis of femur head, Avascular necroses, Non-unions</td>
</tr>
<tr>
<td></td>
<td></td>
<td>• Sinus graft</td>
</tr>
</tbody>
</table>
disease, tumorigenicity). Table 2-2 summarizes the cell types that have been utilized for clinical bone defect repair thus far. Here, I focus on mesenchymal stem cells (MSCs) and endothelial progenitor cells (EPCs) as they have demonstrated to be highly effective in promoting bone and vascular regeneration at bone defects.

### 2.2.2.1. **Mesenchymal Stem Cells**

Mesenchymal stem cells (MSCs) have been long recognized for their potential in engineering bone grafts since they differentiate and form bone during the natural bone development process. Their great potential in BTE has led to their characterization and the identification of a plethora of sources for their isolation. MSCs have been defined through the expression of various CD markers (i.e., negative for CD34, CD45, CD14, CD11a, CD19 and HLA-DR and positive for STRO-1, CD29, CD73, CD90, CD105, CD106, CD166, CD146 and CD44). Also, MSCs have been isolated from a number of adult sources including bone marrow (87), peripheral blood (88), umbilical cord blood (89), synovial membrane (90), deciduous teeth (91), dental pulp (92), amniotic fluid (93), adipose tissue (94), brain, skin, heart, kidneys and liver (95) through a relatively simple protocol that primarily relies on their ability to adhere to tissue culture plastic (96). In addition, their high proliferative potential combined with ability to withstand the freezing conditions allows for their *in vitro* expansion in order to obtain clinically relevant cell numbers (87).

In addition to adult sources, MSCs have recently been derived from embryonic stem cells, as well as iPS cells (97). These embryonic- and iPSC-derived MSCs have the same *in vitro* and *in vivo* multi-potent characteristics as MSCs derived from other adult sources (i.e., bone marrow). However, unlike MSCs derived from adult sources, iPSCs-
derived MSCs may be indefinitely expanded without senescence. Their enhanced survival potential, both \textit{in vitro} and \textit{in vivo}, may be attributed to higher telomerase activity (97). In any case, MSCs of embryonic and iPSC origin have to be further tested to rule out the possibility of teratoma formation before considering them for clinical applications.

The incorporation of MSCs into bone tissue engineering biomaterials is a widely studied strategy for accelerated bone formation and osteointegration during bone defect repair and regeneration. Mechanisms by which enhanced bone regeneration occurs involves directly providing mesenchymal stem cells for osteogenic differentiation and bone formation, as well as enhanced osteoinductivity of the biomaterial via the release of osteogenic growth factors and stimulation of the migration and differentiation of host osteoprogenitors. In addition, pre-differentiating MSCs into the osteogenic lineage before implantation has been shown to further accelerate defect repair and osteointegration of the construct \textit{in vivo} via delivering a more mature osteogenic population capable of immediate bone formation. Pre-clinical trials with MSC-seeded constructs have proven effective in accelerating bone repair in various scenarios including critical-size femoral defects, cranio-maxillofacial deformities, and spinal fusions (98).

Although seemingly a great cellular option for enhanced bone tissue engineering, several issues with their usage have been identified. Firstly, several studies have shown that a maximum of 24–40 population doublings are reached before it comes to a senescence-associated growth arrest. Also, osteogenic differentiation potential \textit{in vitro} and bone forming efficiency \textit{in vivo} significantly decreases with the increasing donor age and systemic disease. Additionally, the lack of knowledge about common markers for
MSCs isolated from different sources make it difficult to define MSCs (99, 100). These factors significantly limit the actual amount and the quality of MSCs obtainable for clinical application. Approximately four to six weeks is required for cell expansion before possible patient treatment. Furthermore, long-term culture may lead to forced selection under artificial culture conditions, which increases the possibility of abnormal karyotype development and malignant cell transformation. Lastly, the use fetal bovine serum (FBS) during in vitro expansion poses a risk of transmitting zoonotic or prion-related diseases, which may induce an immune response triggered by xenogenic proteins. The option of using synthetic serum with range of recombinant growth factors, or serum-free media are being explored as alternatives (86).

At present, a number of strategies have been reported that are capable of augmenting the loss of both proliferative capacity and osteogenic differentiation potential of MSCs after extensive population doublings ex vivo. These methods include cultivation of MSCs in the presence of basic fibroblastic growth factor (FGF-2), and maintenance of MSCs on several extracellular matrices (i.e., basement membrane-like extracellular matrix produced by bovine corneal endothelial cells, denatured collagen type I matrix) instead of conventional tissue culture plastic during progressive number of passages (98). The mechanism of how various ECMs may influence the retention of MSC osteogenic differentiation potential after ex vivo expansion is still ambiguous; however, it has been suggested that the physical interactions between MSCs and certain ECM motifs (i.e., integrins and their ligands) may play a significant role.

The variability of colony formation and culture conditions necessary to sustain proliferative capacity have led to an interesting proposal of the creation of a universal
allogenic human MSC cell line serving as “of-the-shelf” or “ready to use” cells (15). Though it may not seem possible without requiring the use of immunosuppressive drugs to reduce associated risks of rejection, it has recently been shown that cultured MSCs exhibit a poorly immunogenic phenotype (i.e., evidenced by MHC class I+, MHC Class II-, and low level of expression of co-stimulatory molecules, CD40, CD80, and CD86). Also, MSCs have been shown to be immune suppressive (i.e., immune privileged). Specifically, MSCs do not induce the proliferation of lymphocytes, and suppress the proliferation of T-cells and cytokine production in response to alloantigens or insignificant mitogens, as well as inhibiting the function of B cells, dendritic cells and the natural killer cells. This data greatly enhances the therapeutical appeal of MSCs in bone tissue engineering.

2.2.2.2. Endothelial Progenitor Cells

Endothelial cell-mediated vascularization is an absolute pre-requisite for bone tissue formation, repair, and remodeling. Currently, endothelial progenitor cells (EPCs) represent an attractive, if not the most attractive endothelial cell source involved in the repair and angiogenesis of damaged or ischemic bone tissues. In addition, EPCs have been recognized as an attractive autologous endothelial cell population that may easily isolated from peripheral blood or bone marrow in clinic.

EPCs were first characterized in 1997 by Asahara et al. (48). EPCs were defined as bone marrow-derived vascular endothelial cell growth factor-receptor 2 (VEGF-R2) positive, CD43-positive monocyte like cells with the ability to differentiate into endothelial cells in vitro and in vivo based on expression of CD31, eNOS, and E-selectin. EPCs posses a very high and long-term proliferative potential (more than 1,000
population doublings), and may be quickly expanded for clinical use. EPCs display the typical cobblestone morphology of endothelial cells when culture in vitro, and have good angiogenic ability, as demonstrated by complex and intricate network when cultured on and within Matrigel in vitro, and angiogenic abilities in vivo.

Numerous subsequent studies were published attempting to further elaborate on the molecular characterization on EPCs. Other studies demonstrated EPCs to express VEGF-R2, CD133, CXCR-4 receptor, and to posses migrational ability to VEGF and stromal cell derived factor-1 (SDF-1) (101). Other studies suggest that monocyte-like cells expressing CD14, Mac-1 and the dendritic cell marker CD11c demonstrate EPC activity (i.e., uptake of acetylated LDL and binding of ulex-lectin (102, 103). Ingram et al. reported EPC-derived cells express KDR, CD31, and Tie-2 (angiopoietin 1 receptor) (104). Thus, there is still controversy surrounding an accurate molecular definition of EPCs.

The reason for such discrepancy in the molecular definition of EPCs lies in the fact that there is not a universal isolation method being utilized by these studies. There are three common methods that exist for the isolation of EPCs (105). In the first method, peripheral blood mononuclear cells are isolated and plated on fibronectin- or collagen-coated tissue culture plates with a variety of endothelial growth factors. After several days, the non-adherent cells are removed and the adherent cell population remaining, which expresses the ability to ingest acetylated low density lipoprotein and to bind certain plant lectins, is considered to represent EPC. The second method utilizes monoclonal antibodies and fluorescence activated cell sorting (FACS) analysis to enumerate specific cell populations. Asahara et al. reasoned that putative EPC may
express cell surface markers shared by hematopoietic stem cells (HSC) since endothelial and blood cells share a similar mesodermal origin during embryonic development. Thus, Asahara et al. cultured CD34+ cells (15.7% enriched) on fibronectin-coated dishes and observed emergence of spindle shaped cells that expressed a variety of proteins generally expressed by primary endothelial cells. However, EPCs (commonly identified by three cell markers CD133, CD34, and the vascular endothelial growth factor receptor 2) may or may not express CD34, depending on their differentiation state. Specifically, as EPCs are mobilized from the bone marrow into the peripheral blood they begin to lose CD133/CD34 and start to express CD31, vascular endothelial cadherin, and von Willebrand factor (vWF). Therefore, it is critical to identify specific cell markers for EPCs for this method to be effective. The third method involves in vitro colony forming cell assays (i.e., colony forming unit-Hill (CFU-Hill) and endothelial colony forming cell (ECFC) assays). In the CFU-Hill assay, adult peripheral blood or cord blood gives rise to cells that do express many proteins similar to primary endothelial cells, but the CFU-Hill also express numerous myeloid progenitor cell markers and mature into macrophages. Furthermore, CFU-Hill and their progeny fail to spontaneously form human blood vessels when implanted into immunodeficient mice. In contrast, ECFC express cell surface antigens like primary endothelium, clonally propagate and re-plate into secondary and tertiary ECFC, form capillary-like structures in vitro, but most remarkably, form human blood vessels in vivo and inoculate with murine vasculature to become part of the murine systemic circulation. Thus, ECFC display all of the properties of an EPC while the CFU-Hill assay identifies hematopoietic cells.

Although there is still controversy surrounding an accurate molecular definition
and precise phenotype of EPCs, their ability to promote enhanced vascularization and bone formation at bone defects are certain (104). For instance, Yu et al. demonstrated that pre-seeding scaffolds with EPCs effectively promoted neovascularization in grafts, prevented the ischemic necrosis, and improved osteogenesis in a mouse femur bone defect model after 6 weeks post-implantation (47). Zhou et al. and Tan et al. also demonstrated EPC-mediated enhanced vascularization and bone formation in a rabbit bone defect models post-implantation of pre-seeded EPC constructs (45, 49). This observation was also observed in various other animal models including in rats, and sheep (41, 42, 106). Thus, EPCs clearly represent an attractive cell source for promoting bone and vascularization in bone tissue engineering.

2.2.3. Vascularization Techniques

The importance of vascularization to the development and repair of bone tissue has been extensively documented in BTE investigations (107). The greatest amount of newly formed bone occurs in the most vascularized areas, whereas inadequate vascularization at bone defect sites is associated with decreased bone tissue repair and regeneration, and has been identified as the major pitfall to successful BTE. Specifically, until the timely onset of construct vascularization, which is typically on the order of hours to days (i.e., less than 1 mm/day), seeded cells in an implanted BTE construct rely on diffusion for the uptake of nutrients (i.e., oxygen, glucose, etc.) and clearing of metabolic byproducts (i.e., carbon dioxide, lactic acid, etc.), a transport mechanism that is only efficient over short distances (i.e., less than 200 µm) (37). These diffusional constraints result in viable cells located only superficially (i.e., periphery of
the constructs), and thus, limiting the success of the engineering bone tissue throughout the entire thickness of the defect. Although *in vitro* delivery of nutrients to engineered constructs may be alleviated via bioreactor systems, this only delays the diffusional constraint problem to when it is implanted in the host defect site. There is a critical obstacle in maintaining the survival of large masses of cells upon transfer from the *in vitro* culture conditions into the host defect site *in vivo* (108). For this, scientists have proposed several methods to accelerate the onset of neo-vascularization for survival and integration of BTE grafts with host tissue including (1) scaffold design, (2) inclusion of angiogenic growth factors, (3) *in vitro* pre-vascularization (i.e., co-culture of endothelial and osteogenic cells), and (4) *in vivo* pre-vascularization. Although it is still unclear which method is the best for successful *in vivo* application, a combination of these methods may prove to be most effective. The following is a brief review of each method and its challenges.

### 2.2.3.1. Scaffold Design

Scaffold design has a profound effect on the rate of vascularization after implantation. Specifically, mean pore size of the scaffold is a critical determinant of blood vessel ingrowth. BTE studies suggest pore sizes greater than 300 µm to be required for vascular ingrowth (16). Interconnectivity of pore is also critical, as it significantly affects cell migration, and in turn, vascularization. Scaffold fabrication techniques including gas foaming, phase separation and freeze drying are employed in association with porogen leaching for the generation of increasingly porous scaffolds. Recently, the authors developed thermal sintering and porogen leaching method and fabricated
scaffolds with the desired pore size and volume. These scaffolds are proven to be superior because they not only support vascular in growth but also meet the mechanical requirements for bone regeneration (109, 110). On the other hand, methods such as layer-by-layer deposition (i.e., solid free-form fabrication) are now commonly used to actively design scaffold porosity and interconnectivity. With these fabrication systems, production of complex scaffolds with well-defined architecture and optimized pore interconnectivity is possible (111).

### 2.2.3.2. Inclusion of Angiogenic Growth Factors

The local delivery of angiogenic growth factors certainly accelerates vascularization of an implanted graft. Angiogenic growth factors may be incorporated into the BTE construct design either by way of the scaffold or seeded cells. In the first scenario, the growth factor may be incorporated onto the scaffold by (1) simple soaking for resultant fast release, (2) encapsulated in scaffolds, or (3) covalent immobilization for controlled and extended release. Otherwise, growth factors may be incorporated into the seeded cells via genetic modification.

Several critical considerations should be taken into account for success in this method. Firstly, the choice of growth factors is crucial. Several commonly studied angiogenic growth factors in BTE include vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF). Secondly, the proper dosage of the growth factor has been shown to affect the quality of the neo-vasculature. For instance, excess amounts of VEGF have been shown to cause severe vascular leakage and hypotension (112). Lastly, it should be considered that
multiple growth factors along spatial and temporal gradients may allow for even enhanced results as bone tissue development is controlled by the interaction of multiple growth factors. Studies have shown that the incorporation of VEGF and bFGF results in accelerated vascularization of engineered tissues via the mobilization and recruitment of endothelial progenitor cells (EPCs), though the resulting vessels are often disorganized, leaky and hemorrhagic (113). For this reason, the addition of growth factors that stimulate the recruitment of smooth muscle cells or pericytes for the stabilization and maturation of the vessels may be considered, and include PDGF, transforming growth factor-β (TGF-β) and angiopoietin 1 (Ang1) (114, 115).

The addition of growth factors to scaffolds is a relatively easy and widely studied approach. Since this type of growth factor delivery is either driven by passive diffusion or coupled to the rate of biomaterial degradation, the growth factor release may be altered only to some extent by the amount of growth factor added or varying the degradation rate of the material. The release profile for this method is, therefore, often not in tune with the actual healing process and cellular demands (116). On the other hand, growth factors covalently linked to the scaffolds may be released according to the cellular demands. It was demonstrated that the vasculature formed in this manner via controlled release of VEGF formed organized vasculature, in comparison to the vasculature that arose from an uncontrolled VEGF release (117).

The incorporation of growth factors into the scaffolds is not an efficient process. Adding the high price associated with the human recombinant growth factors make the growth factor-loaded scaffold approach not an attractive one. On the other hand, the incorporation of genetically modified cells, such as VEGF releasing ADSCs, has
demonstrated enhanced vascular formation (118). In addition, cells releasing the combination of osteogenic and angiogenic factors (i.e., BMP-4 and VEGF, respectively) together have been shown to increase not only vascular formation, but also the quantity of regenerated bone, compared to when each factor was alternatively delivered alone (119). However, gene therapy in general has safety concerns, and it is not yet approved for clinical use.

2.2.3.3. In Vitro Pre-Vascularization

Current in vitro pre-vascularization strategies of BTE involve the prior seeding and co-culture of endothelial cells and osteogenic cells in BTE constructs in vitro. The mechanism underlying this strategy depends on the direct and indirect communication of these two cell types, and the formation of premature vessels by the endothelial cells in vitro, which may later mature, and anastomose with the host vasculature upon implantation. As seen in Figure 2-5, there is a definite cross-talk between endothelial and osteogenic cells (120). This approach has not only demonstrated accelerated vascularization in vivo, but also enhanced osteogenic differentiation in vitro and bone formation in vivo (43, 121). With this method, anastomoses occurs more quickly in comparison to non pre-vascularized constructs, as host vessels only need to grow into the outer region of the constructs to meet the pre-vascular structures. This method may decrease the time needed for vascularization from weeks to days.
Figure 2-5. Diffusible factors involved in crosstalk between osteoblastic cells and endothelial cells. Growth factors, as well as systemic hormones, can have effects on endothelial functions (purple) and/or on osteoblastic functions (blue). These factors act through activation of specific receptors (black arrows) that in turn stimulate the expression of other proteins after activation of intracellular signaling pathways (green arrows) Adapted from Grellier et al. (120).

An important consideration for in vitro pre-vascularization is the type of utilized cells, and identifying an abundant source of effective autologous endothelial cells. Although mature endothelial cells, isolated from biopsies of skin or saphenous veins may be used, they present with major drawbacks, including insufficient numbers with limited proliferative abilities (i.e., limited in vitro expansion) (122). In contrast, various stem cells, namely endothelial progenitor cells (EPCs) have recently been the topic of discussion. When co-cultured with mesenchymal stem cells and implanted at defect sites,
and constructs demonstrate enhanced levels of vascularization and bone formation. Yu et al. also noted that central necrosis is avoided when scaffolds are seeded with EPCs and MSC-derived osteoblasts, which is not the case when only osteoblasts are seeded alone and implanted (47). Perhaps the most desirable cell source is one that contains both osteogenic and vasculogenic progenitor cells. For instance, mesenchymal stem cells, which may be isolated from bone marrow for osteoprogenitor cells, also have been shown to have the potential to differentiate toward endothelial lineage (123). Another attractive autologous source that may be used to isolate both osteo- and endothelial- progenitors is the stromal vascular fraction (SVF) of adipose tissue, which abundantly available, easy to harvest, and associated with minimal donor site morbidity. In addition, in comparison to bone marrow, it has a much higher frequency of clonogenic mesenchymal progenitors compared (124).

Several issues regarding in vitro pre-vascularization still remain uncertain. For instance, it is unclear whether it is better to maintain the pre-vascularization in vitro long term for establishment of a premature vascular network formation, or to implant the construct shortly after seeding the cells in order to allow the in vivo environment help in the establishment of a functional vasculature. Also, even though endothelial cells have the potential to form new vessels within the scaffolds that may anastomose with host vasculature when implanted in vivo, it is important to consider the presence of other cell types (i.e., smooth muscle cells, pericytes) to ensure the formation of functional vasculature, and so a tri-culture approach should be further investigated (125). Lastly, the potential benefits of this approach have been doubted since it involves cell-containing constructs, which like others require immediate supply with nutrients and oxygen after
implantation. One approach to solve this problem may involve the engineering of vascular axis within the in vitro construct, which can be surgically connected to the host vasculature as it is when vessels are surgically implanted.

### 2.2.3.4. In Vivo (Surgically Induced) Pre-Vascularization

In vivo pre-vascularization may be performed to allow for vascularization of bone constructs. The “flap pre-fabrication” approach utilizes an “extrinsic” mode of vascularization, and involves two main stages. First, the BTE construct is implanted in axially vascularized tissue (i.e., in subcutaneous, intramuscular, or intraperitoneal sites), where microvascular network formation within the constructs occurs within several weeks. The construct is then harvested, and transferred as free bone flap to the bone defect site, where the vascular axis is connected via microsurgical vascular-anastomosis techniques, resulting in instantaneous perfusion of the entire construct. Several drawbacks with this technique include the obvious requirement of two required surgeries, cost, the formation of a random vascularization pattern, degree of vascularization is based on host’s tissue vascularity, as well as donor site morbidity (126). In another method, an “intrinsic” mode of vascularization is used where vessels that are suitable for microsurgical transfer (i.e., carotid artery, jugular vein saphenous bundle, or arteriovenous (AV) loop) are incorporated into the construct (127). Though this procedure has clear advantages over the “flap pre-fabrication” approach, including that it does not require two separate operations like the “flap pre-fabrication” approach, is not dependent upon local vascular conditions and the included vasculature is not randomly oriented, this method is still very challenging since most load-bearing osteogenic constructs are not able to be molded or shaped around the AV loops.
2.3. Functional Bone Tissue Engineering

The term, functional bone tissue engineering, was coined over a decade ago, and is defined as an approach that allows for full functional ability of the graft immediately following their surgical implantation. In this approach, the bone graft to be implanted is required to have carefully defined biomechanical properties to allow its immediate usage upon completion surgery. However, functional bone tissue engineering entails more than just the ability for immediate mechanical usage. Instead, functional bone tissue engineering approaches involve those that allow for the newly restored bone to be fully integrated with the neighboring host bone, and importantly, possess the ability to perform all the functions of native bone. The quality of the new regenerated tissue should seamlessly match that of the host bone. In the future, effective quality assessment tests should be developed to ensure truly functional engineered bone for patients.

2.4. Overall Objective and Specific Aims

Oral/maxillofacial and orthopaedic bone grafts and substitutes represent a combined $2.5 billion market in the United States, a rapidly rising figure due to the aging baby boomer population. Yet, currently available treatment options, including autografts and allografts, are far from ideal. Bone tissue engineering (BTE) is considered one of the most promising treatments on the horizon. Successful BTE critically depends on three components, (i) a three-dimensional, biodegradable scaffold, (ii) an appropriate cell population, and (iii) an adequate vascular supply. We propose the development of an
optimized biodegradable scaffold, seeded with the appropriate cells to promote enhanced bone regeneration and vascularization.

Poly(85 lactide-co-15 glycolide) (PLGA) microsphere scaffolds have attracted significant attention due to their display of better osteocompatibility and human bone-compatible mechanical properties, and the recent approval of PLGA by the US FDA as a biodegradable material for certain biomedical devices. Unfortunately, bone regeneration with these microsphere scaffolds (pore size ~100 µm) is limited to the scaffold surfaces, due to failure to support sufficient mass transport of oxygen and nutrients, osteoclast activity, and neo-vascularization. Recent studies have demonstrated other scaffold types with higher pore sizes (i.e., > 400 µm) can ease these limitations, increase cell infiltration, and ultimately, allow for bone formation and vascularization throughout the entire scaffold. However, as pore size increases, the scaffold’s mechanical strength, a critical BTE factor for load-bearing bones, is sacrificed. Thus, we propose the generation of optimal PLGA microsphere scaffolds with optimally-sized pores (i.e., ~300 µm) to balance the positive and negative features of the previously mentioned pore size ranges, allowing for increased bone regeneration and neo-vascularization, while still retaining human bone-mechanical compatibility.

In addition to an effective biodegradable scaffold, BTE also requires a suitable cell population and sufficient neo-vascularization to accomplish complete bone regeneration. Our proposal includes culturing our optimized scaffolds with two clinically-relevant cell populations for enhanced bone formation and vascularization, specifically peripheral blood derived - endothelial progenitor cells (EPCs) and bone marrow derived - mesenchymal stem cells (MSCs). Previous studies have demonstrated
EPCs and MSCs promote enhanced bone regeneration via the stimulation of neo-vascularization. We will systematically examine the combination of these two cell types and our optimized PLGA scaffolds, and their resultant effects on healing bone defects. We hypothesize that our approach will substantially improve the performance of PLGA microsphere scaffolds, and may offer a practical and effective solution to important clinical problems facing BTE in oral/maxillofacial and orthopaedic surgery.

SPECIFIC AIM 1: To design, fabricate and characterize optimally-porous, mechanically compatible biodegradable microsphere scaffolds.

It is hypothesized that thermal sintering combined with NaCl salt-leaching will generate consistent and reproducible optimally-porous, human bone-mechanically compatible PLGA microsphere scaffolds. In this aim, we will design optimally-porous scaffolds, analyze the porosity via MicroCT, and evaluate mechanical strength via Instron 5544 testings.

SPECIFIC AIM 2: To study the ability of clinically-relevant cell populations to support enhanced mineralization and vascularization on optimally-porous scaffolds in vitro and in vivo.

It is hypothesized that cell seeded - optimally-porous scaffolds will exhibit increased cell survival, proliferation and differentiation of two clinically relevant cell types (i.e., rabbit MSCs and EPCs) as compared to control scaffolds. In this aim, we will seed and culture rabbit MSCs and EPCs, which have been shown to enhance bone and vascular formation,
on the control and optimally-porous at pre-determined ratios in vitro. We will examine the cell survival via a cell viability assay, as well as cellular localization and phenotypic expression via immunofluorescence and confocal microscopy. Also, we will also study the mineralization and vascularization potential of these constructs in mouse subcutaneous model 8 weeks post-implantation.

**SPECIFIC AIM 3:** To assess the ability of optimally-porous biodegradable scaffolds to promote enhanced bone formation and vascularization in vivo.

It is hypothesized that optimally-porous scaffolds will achieve homogeneous bone tissue regeneration via increased neo-vascularization. In this aim, we will implant optimally-porous scaffolds seeded with rabbit MSCs and EPCs in a rabbit ulnar critically-sized bone defect model. At week 12, we will evaluate the explanted grafts via MicroCT to measure bone tissue volume, and histology to assess the enhanced osteogenesis and angiogenesis.

*With these specific aims, we aim to develop pre-vascularized, optimally-porous, human bone-mechanically compatible biodegradable scaffolds for effective bone defect regeneration.*
3. FABRICATION AND IN VITRO EVALUATION OF OPTIMALLY-POROUS MICROSPHERE-BASED SCAFFOLDS

3.1. Introduction

Bone tissue engineering (BTE) has been proposed as a more effective alternative option for bone defect repair/regeneration. BTE involves the combination of biodegradable and porous scaffolds, with or without the use of bone-forming cells and growth factors, to regenerate bone (13, 128). The success of the scaffold-based bone regeneration approach critically depends on the effectiveness of the biodegradable scaffold (129). Important design considerations for BTE scaffolds include biocompatibility, mechanical compatibility, osteoconductivity (i.e., ability of bone cells to grow on scaffold surface and form bone), osteoinductivity (i.e., ability to recruit and stimulate differentiation of progenitor cells into osteoblasts) and osteointegration (i.e., ability to achieve direct bone-to-implant contact) (130). Of these, osteoconductivity of a scaffold is a critical, since cell survival and proliferation in the scaffold’s interior relies on mass transport upon initial implantation until the onset of vascular invasion (131).

Methods to achieve cellular conduction involve scaffold pore structure optimization (16, 17). Scaffolds currently designed with decreased accessible volume and/or small pore sizes (i.e., < 200 μm) display osteoblast survival and bone formation limited to the periphery, due to decreased oxygen and nutrient diffusion throughout the
scaffolds (16). On the other hand, studies have demonstrated that scaffolds with increased accessible volume and/or large pore sizes (i.e., macro-porous, > 200 µm) display increased osteoblast proliferation and differentiation throughout the entire scaffold, due to enhanced mass transport of oxygen and nutrients, and neo-vascularization (23-25). There have been many efforts to improve scaffold overall osteoconductivity by fabricating macro-porous scaffolds that support osteoblast survival and growth throughout the scaffold. Various scaffolding methodologies have been utilized to fabricate macro-porous scaffolds, including gas foaming (28), freeze drying (29, 82), phase separation (30, 83) and porogen leaching (27, 132-134). However, in comparison to scaffolds with smaller pore sizes, these macro-porous scaffolds display a significant decrease in mechanical strength. For instance, Martin et al. demonstrated poly(85 lactide-co-15 glycolide) (PLGA) foam scaffolds to display uniform mineralization throughout, but have inferior mechanical strength (i.e., average compressive modulus of 1.3 MPa) (135). Therefore, there is a clinical need to develop biodegradable scaffolds with optimal pore characteristics and adequate mechanical strength required to support large area bone regeneration.

PLGA microsphere scaffolds have attracted much attention for BTE, because they display human cancellous bone-compatible mechanical properties and have demonstrated bone formation in vivo (31, 32). However, the current PLGA microsphere scaffolds, like other scaffold types with limited accessible volume and pore sizes, fail to provide the prerequisites for optimal bone regeneration (i.e. a stable oxygen and nutrient supply), limiting bone regeneration only to the scaffold surfaces (16, 34). Though Boschetti et al. has reported the fabrication of mechanically-stable macro-porous microsphere scaffolds,
this study included only preliminary cellular biocompatibility studies using fibroblasts (136). In the present study, we systematically examined a novel set of PLGA microsphere scaffolds using pre-osteoblastic cells, and performed comprehensive analyses on the effects of increased porosity and oxygen tension in respect to cell seeding, proliferation, survival, and mineralization. Through this study, we established optimally-porous biodegradable scaffolds that are load-bearing, fully osteoconductive and suitable for large area bone defect repair.

3.2. Materials and Methods

3.2.1. Preparation of PLGA Microspheres

PLGA microspheres were prepared by an oil-in-water method as reported previously (31). In brief, PLGA 85/15 (Lakeshore Biomaterials, Birmingham, AL) was dissolved in methylene chloride (L-14119, Fisher Scientific, Pittsburgh, PA) in a 1:5 dilution ratio (i.e., 4 g PLGA: 20 milliliters of methylene chloride). The PLGA/methylene chloride solution was added slowly to 1 liter of 1% polyvinyl alcohol (PVA, Sigma-Aldrich, St. Louis, MO) solution under a stirring speed of 250 RPM. The stirring continued for 24 hours to allow the methylene chloride to evaporate. The resultant PLGA microspheres were washed with distilled water, filtered, air-dried, sieved into different sizes, and stored in a desiccator until further use.
3.2.2. Fabrication of Optimally-Porous Microsphere Scaffolds

3.2.2.1. Paraffin-Leached PLGA Microsphere Scaffolds

We attempted to fabricate porogen-leached PLGA microsphere scaffolds with paraffin microspheres as the porogen. We created paraffin microspheres using a similar method as that for PLGA microspheres. Briefly, 5 grams of paraffin was melted at 90°C, and then added slowly to 1 liter of 1% polyvinyl alcohol (PVA, Sigma-Aldrich, St. Louis, MO) solution under a stirring speed of 300 RPM. The speed of stirring was increased to a stirring speed of 350 RPM, and ice was added to the mix to decrease the temperature of the stirring solution. The stirring continued for 24 hours. The resultant paraffin microspheres were washed with distilled water, filtered, air-dried, sieved into different sizes, and stored in a desiccator until further use. To fabricate paraffin-microsphere PLGA microsphere scaffolds, we mixed PLGA microspheres (diameter 425-600 µm) and a porogen, paraffin (diameter 200-300 µm), were mixed at specific weight ratios (i.e., PLGA:paraffin ratios of 100:0, 90:10, 80:20, 70:30, 60:40 and 50:50). The PLGA/paraffin mixture was then placed into a steel mold, and thermally sintered at 100°C. At this time, we observed collapsed scaffold forms since the paraffin melted during the sintering process. We then turned to NaCl crystals for a superior porogen for the fabrication of PLGA microsphere scaffolds with increased porosity.

3.2.2.2. Salt-Leached, Optimally-Porous PLGA Microsphere Scaffolds
A new scaffolding method of “Thermal Sintering and Porogen Leaching” developed in this study was used to fabricate microsphere scaffolds with increased porosity. A schematic illustration of this method is shown in Figure 1a. In brief, PLGA microspheres (diameter 425-600 μm) and a porogen, NaCl (diameter 200-300 μm), were mixed at specific weight ratios (i.e., PLGA:NaCl ratios of 100:0, 90:10, 80:20, 70:30, 60:40 and 50:50). The PLGA/NaCl mixture was then placed into a steel mold, and thermally sintered at 100°C. The NaCl porogen was leached out by soaking the composite PLGA/NaCl scaffolds in distilled water for 2 hours, resulting in scaffolds with increased porosity compared to control scaffolds. The scaffolds with 0% NaCl are referred to as control PLGA scaffolds, while the rest as macro-porous scaffolds. We fabricated disc-shaped scaffolds (10 mm diameter, 2 mm height) for porosity measurements and the majority of cellular studies, and cylinder-shaped scaffolds (5 mm diameter, 10 mm height), which were utilized for mechanical testing, live/dead study, and part of mineralization studies. Scaffolds were air-dried and stored in a desiccator until future use. Scanning electron microscopy (SEM) was used to image the morphology of the microsphere scaffolds and visually examine the increased number of large pore sizes after NaCl-leaching.

3.2.3. Morphology of Optimally-Porous PLGA Microsphere Scaffolds

3.2.3.1. Scanning Electron Microscope Analysis

Scanning Electron Microscope (SEM, FEI Strata 400s Dual Beam FIB) analysis
was utilized to examine the morphology of the fabricated PLGA microsphere scaffolds (i.e., control scaffold, composite PLGA microsphere/NaCl scaffold before and after salt leaching). Samples were prepared by coating with gold/palladium and examined under SEM.

3.2.3.2. Micro-Computed Tomography Analysis

Scaffold specimens were imaged using cone beam micro-focus X-ray computed tomography to render three-dimensional models for direct quantitation of porosity (µCT40, Scanco Medical AG, Bassersdorf, Switzerland). Serial tomographic images were acquired at 45 kV and 177 µA, collecting 2000 projections per rotation at 300 msec integration time. Three-dimensional 16-bit grayscale images were reconstructed using standard convolution back-projection algorithms with Shepp and Logan filtering, and rendered within a 12.3 mm field of view at a discrete density of 4,629,630 voxels/mm3 (isometric 6 µm voxels). Segmentation of solid scaffold from open porosity was performed in conjunction with a constrained Gaussian filter to reduce noise, applying a threshold of -220 Hounsfield units (water = 0, air = -1000). Direct measurements of internal porosity included volume fraction, size, connectivity, accessible internal pore volume, and accessible solid surface area of scaffold (as a function of pore dimension). The accessible volume and surface parameters provide direct measurements of the pore volume and surface available to cell infiltration as a function of minimum pore dimension, using a distance transformation algorithm similar to Moore et al. (137)
3.2.4. Mechanical Evaluation of Optimally-Porous PLGA Scaffolds

Compressive testing of cylindrical PLGA microsphere scaffolds (5 mm diameter x 10 mm height, n=6/group) was performed at 2 mm/min (model 5544, Intron Corp., Norwood, MA) following the standard protocol of ASTM 1621 (138). Compressive strength was defined as the maximum stress magnitude. Apparent modulus was measured as the tangential slope of the linear region of the effective stress-strain curve at 50% of compressive strength magnitude.

3.2.5. In Vitro Evaluation of Optimally-Porous PLGA Scaffolds

3.2.5.1. Pre-Osteoblast MC3T3-E1 Cell Culture and Evaluation on Optimally-Porous PLGA Scaffolds

3.2.5.1.1. MC3T3-E1 Cell Seeding and Culture on Optimally-Porous PLGA Scaffolds

The pre-osteoblast immortalized cell line MC3T3-E1 (American Type Culture Collection, Manassas, VA) were cultured in α-minimal essential medium (α-MEM) containing 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C, 5% CO2 and 95% humidified air. Cells were maintained in sub-confluent cultures until needed for in vitro scaffold studies.
PLGA microsphere scaffolds were sterilized by immersing the scaffolds in 70% ethanol for twenty minutes. Scaffolds were then washed three times in sterile PBS before exposing them to UV radiation for one hour. After cell trypsinization, a MC3T3 cell suspension containing 4 x 10^4 cells was uniformly seeded onto the scaffolds. The disc-shaped scaffolds were placed flat on the culture plate, and a 20 µl cell suspension was uniformly added to the top of the scaffold. The cylinder-shaped scaffolds were placed along the length of the scaffold on the culture plate, and a 40 µl cell suspension was added to the lengthwise surface as the scaffold was slowly rotated (i.e., along the long axis of the scaffold) to maintain uniform cell seeding. The cell-seeded scaffolds were incubated for two hours at 37°C to allow for cell adhesion onto the scaffolds. The cell-scaffold constructs were cultured in osteogenic media (i.e., α-MEM supplemented with 10% FBS, 1% penicillin-streptomycin, 3mM β-glycerophosphate and 50 µg/ml ascorbic acid), and maintained for 7, 14, 21, and 28 days in an incubator at 37°C, 5% CO₂, and 95% humidified air.

3.2.5.1.2. MC3T3-E1 Cell Seeding Efficiency on Optimally-Porous PLGA Scaffolds

After 6 hours of cell seeding, scaffolds were transferred to new wells. Cells at the bottom of the original wells were trypsinized, resuspended and counted with a hemacytometer. Cell seeding efficiency (i.e., the number of cells that adhered to the scaffolds) was determined by the difference between the number of cells initially seeded (i.e., 4 x 10^4 cells) and the number of cells that were counted at the bottom of the well.
3.2.5.1.3. MC3T3-E1 Cell Proliferation on Optimally-Porous PLGA Scaffolds

DNA concentration of the pre-osteoblast MC3T3 cells cultured on control PLGA scaffolds and PLGA scaffolds with increased porosity (n=3) was evaluated quantitatively using Quant-iT PicoGreen dsDNA assay (Invitrogen, Carlsbad, CA) following manufacturer’s instructions. After culturing the samples (scaffold dimensions 8 mm diameter x 2 mm height) for 7, 14, and 21 days in osteogenic media, the cell-scaffold samples (n=3) for each experimental group were harvested. Samples were washed with PBS, incubated in lysis buffer (i.e., 1% Triton X-100 solution), and subjected to freeze–thaw cycles. DNA concentration from the cell lysates was determined according to the manufacturer’s protocol. Experimental groups included control scaffolds (i.e., 0:100 ratio of NaCl:PLGA), and scaffolds with increased porosity (i.e., 10:90, 20:80, 30:70, and 40:60 ratio of NaCl:PLGA).

3.2.5.1.4. MC3T3-E1 Cell Viability on Optimally-Porous PLGA Scaffolds

The live-dead cell viability assay we used (Invitrogen, Carlsbad, CA) includes calcein AM and ethidium homodimer-1 probes to label live and dead cells green and red, respectively. We used this live-dead assay to compare MC3T3 cell survival on the surface and in the interior of scaffolds. Cylindrical scaffolds (5 mm diameter, 10 mm height) were cultured for 4, 7, and 14 days in osteogenic media, at which point the scaffolds (n=3) for each experimental group were harvested. Samples were bisected
lengthwise from each scaffold group to allow for the examination of cell viability in the sample’s interior. Live-dead cell viability assay was performed according to the manufacturer’s protocol, using confocal microscopy to image cells at the surface and interior of the scaffolds.

3.2.5.1.5. MC3T3-E1 Cell Localization and Expression on Optimally-Porous PLGA Scaffolds

To visualize cellular localization and expression via histology and immunohistochemistry, samples were paraffin-embedded and sectioned (139). Briefly, cell-scaffold constructs were washed with PBS, and then fixed in formalin overnight at 4°C. Samples were dehydrated sequentially using an isopropyl alcohol series (i.e., 70%, 90% and 100%) for one hour each, at room temperature. Samples were directly transferred to molten paraffin (Tissue Path Paraplast Tissue Embedding Media, Fisher Scientific, Pittsburgh, PA) at 55°C for 10 minutes and then embedded in fresh molten paraffin. Paraffin-embedded samples were cut into serial sections (20 µm thick) using Cryofilm (Section-Lab Co. Ltd., Hiroshima, Japan) and a microtome sectioning machine. Sections were placed on glass slides for histological analysis. Sections were stained with Gill’s 3 hematoxylin (Sigma-Aldrich, St. Louis, MO) to visualize MC3T3 cell localization within the PLGA scaffolds after culturing the constructs for 28 days in osteogenic media. Immunostaining of two bone markers, osteopontin (OPN) and collagen Type I (Col I) was performed via a rabbit polyclonal anti-human osteopontin (Abcam, Cambridge, MA, ab8448) antibody and a rabbit polyclonal anti-human collagen type I antibody (Abcam, Cambridge, MA, ab292), respectively. Briefly, sections were de-
paraffinized in HistoClear (National Diagnostics, Atlanta, Georgia), taken through a descending series of ethanol concentrations, rehydrated in distilled water, and then placed in 3% hydrogen peroxide (Sigma-Aldrich, St. Louis, MO) in PBS to quench endogenous peroxidase activity. To improve antigen exposure, the sections were boiled in Target retrieval (Dako, Carpinteria, CA) and washed in distilled water. Samples were incubated with blocking solution (i.e., 0.5% (w/v) bovine serum albumin in PBS) for one hour. Primary antibodies were diluted in 0.5% (w/v) bovine serum albumin in PBS at concentrations of 1:200 for OPN or 1:300 for Col I. Samples were incubated with the primary antibody for two hours at room temperature. Sections were washed free of primary antibody and incubated with SignalStain® Boost IHC Detection Reagent, HRP, Rabbit (Cell Signaling, Boston, MA, #8114) followed by an incubation with 3,3'-diaminobenzidine (DAB, Vector Laboratories, Burlingame, CA) for thirty seconds. The slides were rinsed three times in water, and mounted using mounting media (Electron Microscopy Sciences, Hatfield, PA) for thirty minutes.

3.2.5.1.6. MC3T3-E1 Cell Mineralization on Optimally-Porous PLGA Scaffolds

Matrix mineralization or calcium deposition was evaluated via Alizarin Red staining. This colorimetric analysis is based on solubility of the red matrix precipitate with cetylpyridinium chloride (CPC, Sigma-Aldrich, St. Louis, MO) to yield a purple solution. Briefly, after 14, 21 and 28 days of culturing in osteogenic media, cell-scaffold constructs were washed with distilled water and fixed with 70% ethanol at 4°C for one hour. Ethanol was removed and samples were air-dried for ten minutes. Samples were
incubated with alizarin red dye (Sigma-Aldrich, St. Louis, MO) for ten minutes at room temperature. Following washes to remove excess alizarin red dye, the samples were incubated with 10% CPC at room temperature for thirty minutes. The absorbance of the resulting solution, which is proportional to the amount of calcium deposited, was read on a TECAN plate reader at 562 nm.

3.2.5.1.7. Oxygen Tension Measurements on Optimally-Porous PLGA Scaffolds

After culturing the MC3T3-E1 cells on control and optimally-porous PLGA scaffolds for 21 days in osteogenic media, the cell-scaffold samples were quantitatively evaluated for the oxygen tension in the interior region of each sample group using needle-type fiber optic oxygen microsensors (501656, World Precision, Saratoga, FL), as previously described by Volkmer et al. (25). Specifically, we examined scaffold groups with 0%, 10%, 20%, 30%, and 40% NaCl, and cultured 100,000 cells on each scaffold (dimensions 5 mm diameter, 10 mm height). The oxygen sensors were mounted on optic fibers with a tip diameter of 50 µm. To protect these fragile sensors, they are fixed within a standard hollow 27 gauge needle of 0.4-mm diameter. A 25 gauge needle was utilized to pre-form a 2.5 mm deep channel on the side of the scaffold for which the probe would then be inserted (305127, Becton Dickinson). Oxygen tension measurements in the medium were carried out by inserting a probe in the medium next to all experimental scaffold groups. Prior to sample measurements, the oxygen microsensor was calibrated following a conventional two-point calibration protocol described by the manufacturer. Briefly, oxygen-free water and water-vapor saturated air were used as
calibration standards. The oxygen-free water standard was prepared by dissolving one gram of sodium sulfite (S430, Fisher Scientific) in 100 milliliters of water in a sealed vessel, and the water-vapor saturated air was prepared by placing a wet piece of cotton in a sealed vessel. The oxygen tension measurements were obtained by inserting the probe mid-length and mid-diameter (Figure 3-1). Measurements are expressed as the mean of three samples per scaffold group ± standard deviation.
Figure 3-1. A) Photograph of needle-type fiber optic oxygen / pH microsensors set-up. (B) Photograph of needle-type fiber optic micro-sensors inserting into the interior region the cell-scaffold construct.

3.2.5.2. Mesenchymal Stem Cell (MSC) Culture and Evaluation on Optimally-Porous PLGA Scaffolds
3.2.5.2.1. MSC Isolation

Mesenchymal stem cells (MSCs) were isolated from the bone marrow of one New Zealand white rabbit (4-5 kg). The mononuclear cell fraction was isolated via layering over a Percoll density gradient, and centrifuging at 600 rpm for 20 min at room temperature. The mononuclear cell fraction was seeded and expanded in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% FBS and 1% Penicillin/Streptomycin (P/S, Gibco) at 37°C and 5% CO₂. Passages 3-5 were used for experimentation.

3.2.5.2.2. MSC Seeding and Culture on Optimally-Porous PLGA Scaffolds

PLGA microsphere scaffolds (10 mm x 5 mm) were sterilized by immersing the scaffolds in 70% ethanol for twenty minutes. Scaffolds were then washed three times in sterile PBS before exposing them to UV radiation for one hour. After cell trypsinization, a MSC cell suspension was uniformly seeded onto the scaffolds. The cylinder-shaped scaffolds were placed along the length of the scaffold on the culture plate, and a 40 µl cell suspension of 1 x 10⁵ cells was added to the lengthwise surface as the scaffold was slowly rotated (i.e., along the long axis of the scaffold) to maintain uniform cell seeding. The cell-seeded scaffolds were incubated for two hours at 37°C to allow for cell adhesion onto the scaffolds. The cell-scaffold constructs were cultured in osteogenic media (i.e., α-MEM supplemented with 10% FBS, 1% penicillin-streptomycin, 3mM β-
glycerophosphate and 50 µg/ml ascorbic acid), and maintained for 7, and 21 days in an incubator at 37°C, 5% CO₂, and 95% humidified air.

3.2.5.2.3. MSC Cell Viability on Optimally-Porous PLGA Scaffolds

Live-dead cell viability assay (Invitrogen, Carlsbad, CA) was used to analyze cell survival in the interior of cell-seed constructs. We seeded and cultured 100,000 MSCs on scaffolds (0% NaCl/100% PLGA, and 20% NaCl/80% PLGA) in osteogenic media media (i.e., α-MEM supplemented with 10% FBS, 1% penicillin-streptomycin, 3mM β-glycerophosphate and 50 µg/ml ascorbic acid) for 7 and 21 days 37°C and 5% CO₂. Samples were bisected lengthwise from each scaffold group (n=3) to allow for the examination of cell viability in the sample’s interior. Live-dead cell viability assay was performed according to the manufacturer’s protocol to label live cells green with calcein AM, and dead cells red with ethidium homodimer-1 probes. Confocal microscopy (Zeiss LSM ConfoCor2, 20X magnification) was utilized to image cells interior of the scaffolds.

3.2.5.2.4. Oxygen Tension Measurements on Optimally-Porous PLGA Scaffolds

Needle-type fiber optic oxygen microsensors (501656, World Precision, Saratoga, FL) were utilized to analyze oxygen levels in the interior of MSC-seeded control and macro-porous scaffolds (25). Briefly, 100,000 MSCs were seeded on each scaffold type (i.e., 0% NaCl, 10% NaCl, 20% NaCl, 30% NaCl, 40% NaCl), and cultured for 21 days
in osteogenic media (DMEM, 10% FBS, 10 nM dexamethasone, 50 µg/ml ascorbic acid, 5 mM β-glycerophosphate) 37°C and 5% CO₂. At this point, a 25 gauge needle was utilized to pre-form a 2.5 mm deep channel on the side (mid-length) of the scaffold for which the probe would then be inserted (305127, Becton Dickinson). Prior to sample measurements, the oxygen microsensor was calibrated following a conventional two-point calibration protocol described by the manufacturer. Briefly, oxygen-free water and water-vapor saturated air were used as calibration standards. The oxygen-free water standard was prepared by dissolving one gram of sodium sulfite (S430, Fisher Scientific) in 100 milliliters of water in a sealed vessel, and the water-vapor saturated air was prepared by placing a wet piece of cotton in a sealed vessel. Oxygen tension measurements in the medium were carried out by inserting a probe in the medium next to all experimental scaffold groups. Oxygen tension measurements of the interior of the cell-seeded scaffolds was carried out by placing the probe tip in the center of the scaffold by way of the pre-formed channel made in the constructs. Oxygen tension measurements are expressed as the mean of three samples per scaffold group ± standard deviation.

3.2.5.2.5. pH Measurements on Optimally-Porous PLGA Scaffolds

Needle-type fiber optic pH microsensors (World Precision, Saratoga, FL) were utilized to analyze pH levels in the interior of MSC-seeded control and macro-porous scaffolds (25). Cell seeding on scaffolds and culture in vitro was performed in same manner for the oxygen tension measurement samples. Prior pH measurements, the pH microsensors were calibrated with buffer solutions of pH 5.0, 6.0, 7.0 and 8.0. pH measurements in the medium were carried out by inserting a probe in the medium next to
all experimental scaffold groups. pH measurements of the interior of the cell-seeded scaffolds was carried out by placing the probe tip in the center of the scaffold by way of the pre-formed channel made in the constructs. pH measurements are expressed as the mean of three samples per scaffold group ± standard deviation.

### 3.2.6. Statistical Analysis

For scaffold porosity analysis, cell seeding efficiency, cell proliferation, mineralization, and oxygen tension and pH measurement analyses, a two-way analysis of variance (ANOVA) was performed to compare data. Three scaffolds per group were analyzed at each time point. Error is reported in figures as the standard deviation (SD) and significance was determined using a probability value of p < 0.050.

### 3.3. Results

#### 3.3.1. Microsphere Scaffolds with Increased Porosity

![Diagram of microsphere scaffold fabrication process]

**Figure 3-2.** (A) Schematic diagram illustrating the fabrication process of PLGA microsphere scaffolds with increased pore sizes. SEM image of (B) PLGA microspheres after thermal sintering, (C) PLGA/NaCl composite scaffold after
sintering, and (D) PLGA scaffold with increased pore sizes created after thermal sintering and porogen leaching.

PLGA microsphere sintering often results in three-dimensional scaffolds with limited pore volume. However, we developed a novel method to fabricate PLGA microsphere scaffolds with increased pore volume and average pore size, as shown in Figure 3-2. Through mixing a porogen (i.e., NaCl crystals, 200-300 µm diameter) with PLGA microspheres (425-600 µm diameter), thermal sintering, followed by porogen leaching, we successfully created PLGA microsphere scaffolds with increased pore volume. Thermal sintering, and particulate leaching are well known methods for fabricating three-dimensional and porous scaffolds (31, 132). Here, we combined them into a single method “Thermal Sintering and Porogen Leaching” to design PLGA microsphere scaffolds with the desired pore characteristics. SEM imaging demonstrated that after porogen leaching (Figure 3-2D), there was visually an increase in number of large pore sizes compared to scaffolds fabricated with PLGA microspheres alone. (i.e., control PLGA scaffolds).

3.3.2. Scaffold Porosity via Micro-CT

MicroCT imaging was used to reconstruct 3D models of scaffolds for nondestructive measurements of porosity. Computational assessment of all MicroCT images confirmed that the internal porosity is one interconnected space comprising 99.9% of the total pore volume. The porosity and accessible volume of the PLGA microsphere scaffolds corresponded to porogen size and amount used. PLGA microsphere scaffolds that were not fabricated with NaCl (i.e., control scaffolds), displayed a void volume of approximately 39 mm³, whereas scaffolds fabricated with
20% NaCl and 40% NaCl (percent by weight) displayed a void volume of 50 mm$^3$ and 59 mm$^3$, respectively (Figure 3-3). Also, by mixing 40% NaCl and 60% PLGA microspheres (by dry weight), percent accessible pore volume increased 337% in relation to control scaffolds at an average pore size of 200 µm (Figure 3-4B). Data and images describing scaffold pore volume are presented as a function of pore size, providing direct measurements of externally accessible pore space through the full range of diametral pore dimension (Figure 3-4A, C). Although the range of pore size dimensions remained constant, the volume of porosity increased with higher concentration of porogen. For example, blue areas signify the accessible volume in the scaffolds to objects with a diameter in the range of 100-200 µm, and red in the range of 400-500 µm. Thus, as porogen concentration increased, the accessible interconnected volume also increased (Figure 3-4A). In control scaffolds, a sphere with a diameter of 200 µm can access approximately 10% of the total pore volume, whereas the same sphere can access approximately 40% of the pore volume of the 40% NaCl/60% PLGA scaffold (Figure 3-4B). This is illustrated in Figure 3-5C, which again shows that the experimental scaffolds fabricated with a porogen have a higher percentage of accessible pore volume in the 300-500 µm range in comparison to control scaffold. Thus, we effectively increased the accessible volume for cell infiltration throughout the scaffold. In addition, as seen in Figure 3-5, scaffolds fabricated with at least 20% NaCl demonstrated significantly enhanced pore sizes in the range of 300-400 µm as compared to control matrixes (i.e., 0% NaCl).
Figure 3-3. Increasing porosity (i.e., void volume (mm$^3$)) in PLGA microsphere scaffolds fabricated with a porogen (NaCl).

<table>
<thead>
<tr>
<th>Void Volume (mm$^3$)</th>
<th>0% NaCl</th>
<th>10% NaCl</th>
<th>20% NaCl</th>
<th>30% NaCl</th>
<th>40% NaCl</th>
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<tbody>
<tr>
<td></td>
<td>39.1</td>
<td>40.7</td>
<td>49.5</td>
<td>52.1</td>
<td>59.3</td>
</tr>
</tbody>
</table>
Figure 3-4. Scaffold pore interconnectivity and percent accessible volume obtained via micro-computed tomography imaging and analysis. (A) Accessible volume space images generated by imposing specific pore diameter parameters (scale 100–500 mm) on 0% NaCl/100% PLGA, 20% NaCl/80% PLGA, and 40% NaCl/60% PLGA scaffolds from a top-view, mid-view, and cross-sectional view. (B) Graph comparing the effect of increasing porogen to accessible volume in the PLGA scaffolds. Dashed
line illustrates percent accessible volume of PLGA/0% NaCl and PLGA/40% NaCl scaffolds for an object with a diameter of 200 mm. (C) Increasing scaffold accessible volume in optimally-porous PLGA scaffolds. Interconnected volume accessible to spherical objects with a specific diameter range (i.e., 100–200, 200–300, 300–400, and 400–500 mm) in PLGA/0% NaCl, PLGA/20% NaCl, and PLGA/40% NaCl scaffolds.

Figure 3-5. (A) Percent accessible volume of each scaffold group in the range of 200–400 µm. (B) Histogram of percentage of pores in increments of 100 µm for each scaffold group. (C) Graphs illustrating percentage of pores within each pore size range.
3.3.3. Scaffold Mechanical Characterization

By using an increasing amount of porogen in the scaffold fabrication process, compressive strength and modulus of the scaffolds were sacrificed (Figure 3-6). Scaffolds with increased porosity (i.e., 40% NaCl/60% PLGA by dry weight) displayed significantly less compressive strength and modulus than control scaffolds (i.e., 0% NaCl/100% PLGA). Scaffolds with optimally-sized pores (i.e., 20% NaCl/80% PLGA) displayed a significant decrease, 63.2%, in compressive strength, and a 29.8% decrease in compressive modulus in comparison to control scaffolds. 20% NaCl/80% PLGA displayed significantly higher compressive strength, 140%, and modulus, 240%, than scaffolds with the highest porosity (i.e., 40% NaCl/60% PLGA). The compressive modulus and strength for the scaffolds with increased porosity (i.e., 10-40% NaCl/90-60% PLGA), although lower than the control scaffold, are in the range of human trabecular bone mechanical properties (i.e., compressive modulus 50-800 MPa and compressive strength 1-10 MPa. Thus, we termed the 20% NaCl/80% PLGA scaffold group as optimally-porous scaffolds, since they display significantly higher pore volume than control, while retaining mechanical strength.
Figure 3-6. Mechanical characterization of PLGA microsphere scaffolds with an increased porogen content. Analysis of (A) compressive strength and (B) compressive modulus.

3.3.4. Effect of Scaffold Accessible Pore Volume on In Vitro Cell Performance

3.3.4.1. MC3T3-E1 Cell Performance on Optimally-Porous PLGA Scaffolds

3.3.4.1.1. MC3T3-E1 Cell Infiltration, Proliferation and Survival

The efficiency of initial cell seeding decreased with increasing porogen used to fabricate the PLGA microsphere scaffolds (Figure 3-7). Of the $4 \times 10^4$ MC3T3 cells initially seeded onto each scaffold, approximately $3.3 \times 10^4$ cells adhered to the control scaffolds, and only $2.5 \times 10^4$ cells adhered to the scaffolds with increased porosity (i.e., 40% NaCl/60% PLGA by dry weight). However, after 5 days of culture in osteogenic media, the DNA concentration, which is proportional to cell number, was not significantly different in control scaffolds and scaffolds with increased porosity (i.e., 20% NaCl/80% PLGA and 40% NaCl/ 60% PLGA by dry weight). By 2 weeks of culture, cell number and proliferation in the scaffolds with increased porosity exceeded that of control scaffolds (Figure 3-8). The limitation of cell culture on scaffolds was seen by 3 weeks of culture, as the capacity of the scaffolds to support cell proliferation began to decrease. The effects of scaffold porosity on cell viability were examined on the surface of the scaffolds, as well as the interior, of the scaffolds. At 4, 7, and 14 days of cultures, cell-scaffold constructs were bisected, and live/dead assays were performed. Representative fields from the center of the scaffold (approximately 5 mm depth) taken
by confocal microscopy are shown in Figure 3-9. After 4 days of culture, there was not a significant difference in live : dead cell ratio between the control scaffolds and scaffolds with increased porosity. By 14 days of culture, we observed a significant difference in live cells present in the interior region of scaffolds with increased porosity versus control scaffolds. In 20% NaCl/80% PLGA and 40% NaCl/ 60% PLGA scaffolds, the cells displayed a robust and healthy morphology, with extended processes. In contrast, the cells in the center of the control scaffolds were mostly dead by 14 days and displayed a round morphology appearance.

Figure 3-7. Effect of increasing porosity on cell-seeding efficiency on scaffolds (*p < 0.05).
Figure 3-8. Effect of increasing porosity on proliferation of murine pre-osteoblast cells (MC3T3-E1) seeded on PLGA control and optimally porous scaffolds at 5, 14, and 21 days (*p < 0.05).
Figure 3-9. Effect of increasing porosity on cell viability in the interior of the PLGA microsphere scaffolds at 4, 7, and 14 days (scale = 200 mm).

3.3.4.1.2. MC3T3-E1 Cell Localization and Expression

Through a modified paraffin-embedding and sectioning procedure, we were able to study the cellular localization and expression of the MC3T3 cells cultured on our PLGA microsphere scaffolds. In Figure 3-10, PLGA scaffolds with increasing porosity (i.e., 20% NaCl/80% PLGA and 40% NaCl/60% PLGA; images of 40% NaCl/60% PLGA scaffolds are not shown) promote cell infiltration into the interior of the scaffolds. After 28 days of culturing MC3T3 cells on the scaffolds, hematoxylin staining highlighted cells densely located on the top of control scaffolds and not in the center of the control scaffolds (Figure 3-10A). On the other hand, scaffolds with increased
porosity displayed cell localization on the surface, as well as increased cell infiltration and survival in the center of the scaffold (Figure 3-10 B, C). Likewise, we found cells expressing osteopontin and collagen type I only on the surface of the control scaffolds (Figure 3-10 D, G). PLGA scaffolds with increased porosity displayed cells expressing osteopontin and collagen type I at the top, as well as the center of the scaffold (Figure 3-10 E, F, H, I).

**Figure 3-10.** MC3T3-E1 cellular localization and expression on control and optimally porous PLGA scaffolds. Hematoxylin staining of control (A) and
optimally porous scaffolds [top (B) and center (C)], osteopontin immunohistochemistry of control (D) optimally porous scaffolds [top (E) and center (F)], and collagen type I immunohistochemistry of control (G) and optimally porous scaffolds [top (H) and center (I)]. Arrows indicate cell staining. Scale on all images = 200 µm.

3.3.4.1.3. MC3T3-E1 Cell Mineralization

After 28 days following seeding and culturing MC3T3 cells on scaffolds, we performed Alizarin Red Staining to detect calcium mineralization. Scaffolds with increased porosity visually appeared to have higher mineralization potential than control scaffolds (Figure 3-11). Control scaffolds displayed mineralization limited to the top surface, and not in the center and bottom. On the other hand, scaffolds with increased porosity showed Alizarin red staining throughout the entire scaffold (i.e. top and bottom surfaces, and middle of construct). To compare and quantify the mineralization that was occurring throughout the scaffolds versus mineralization occurring only in the center of the scaffolds, we cultured MC3T3 cells on cylindrical scaffolds that were taller (scaffold size 10 mm height, 5 mm diameter), so that we were able to manually dissect 2 mm off the top and bottom scaffold surfaces (Figure 3-12). Alizarin Red staining quantification confirmed the increase in mineralization in scaffolds with increasing porosity. 20% NaCl/80% PLGA scaffolds displayed the highest significant difference in mineralization compared to control. Although mineralization in 40% NaCl/60% PLGA scaffolds displayed higher mineralization potential than control scaffolds, it was not as high as 20% NaCl/80% PLGA (Figure 3-12 A). After manually removing the top 2 mm and bottom 2 mm surfaces of the tall cylindrical scaffolds, we quantified the mineralization in the center portions of the scaffolds. Mineralization increased significantly in the center of the scaffolds with increasing porosity after 28 days in culture (Figure 3-12 B).
Figure 3-11. Mineralization potential of control and optimally-porous PLGA microsphere scaffolds. Alizarin red staining was performed 28 days after MC3T3-E1 cells had been cultured on scaffolds. Red staining in images signifies mineralization or calcium deposition. Optimally porous scaffold displayed mineralization throughout the scaffold (i.e., top and bottom surfaces, and cross section), while control scaffold mineralization is limited to only the top surface of the scaffold. Scale bar = 1000 µm.
Figure 3-12. Effects of porosity on mineralization (A) throughout the entire scaffold, and (B) in the center of the scaffold. After 28 days of cell culture on scaffolds, Alizarin Red staining was performed on the entire cell-scaffold construct and quantified. Two millimeters from the top and bottom surfaces of the scaffolds were manually removed as shown in (C), and Alizarin red staining was performed to analyze the mineralization in the center of the constructs (B) (* signifies p < 0.05). Photographs of control scaffolds (0% NaCl/100% PLGA) seeded without cells and with MC3T3-E1 cells, and optimally-porous scaffolds (20% NaCl/80% PLGA scaffolds) seeded with MC3T3 cells. Mineralization in control scaffold is limited to the top of the scaffold, whereas mineralization of optimally-porous scaffolds extends significantly lower than control scaffolds (Scale bar = 5 mm).

3.3.4.1.4. Oxygen Tension Levels in Scaffold’s Interior Regions

Oxygen tension measurements demonstrated a significant gradient between the media surrounding the cultured constructs and the interior regions of the constructs in all experimental groups after 3 weeks in vitro. Oxygen tension in the peri-construct region
for all experimental scaffold groups was not statistically different from each other, and averaged 6.67% ± 1.11%. Oxygen tension in the interior of the cell-seeded scaffolds was directly related to the concentration of porogen used to fabricate the scaffolds. Specifically, increases in the scaffold’s porosity facilitated and enhanced oxygen diffusion to the construct’s interior region, and thus, decreasing the oxygen tension gradient from the scaffold’s exterior to interior (Figure 3-13). The peri-construct – interior construct oxygen gradient was most significantly seen in control scaffolds, where the oxygen gradient in the interior of cell-seeded control (0% NaCl/100% PLGA scaffolds) scaffolds conditions dropped below 1%. Optimally-porous scaffolds (20% NaCl/80% PLGA scaffolds) displayed similar oxygen tension gradients as compared to macro-porous scaffolds (40% NaCl/60% PLGA scaffolds).
Figure 3-13. The effect of porosity on oxygen tension gradient from the exterior to interior of PLGA microsphere scaffolds after 3 weeks in vitro. Oxygen tension of ambient air is 21%, and average oxygen tension of cell culture media is 6.67% – 1.11%.

3.3.4.2. **MSC Performance on Optimally-Porous PLGA Scaffolds**

3.3.4.2.1. MSC Viability

*In vitro* culture of mesenchymal stem cells (MSCs) on optimally-porous microsphere scaffolds resulted in enhanced cell survival throughout the entire construct, compared to that of control scaffolds over long-term 21 day culture. As seen in Figure
3-14, optimally-porous scaffolds cultured with MSCs displayed significantly more live cells (i.e., live cells are stained green with calcein AM, dead cells are stained red with ethidium bromide) in the interior of the construct, whereas control constructs displayed significant cell death in the interior regions of the construct after 21 days in vitro. This observed increasing cell survival trend can be attributed to the increased oxygen tension levels, as well as more normal pH levels in the interior of the scaffolds, as compared to that of control scaffolds after 21 day in vitro.

Figure 3-14. MSC viability in interior of control and optimally-porous scaffolds at 7 and 21 days in vitro. Live cells fluoresce green (i.e., calcien-AM), and dead cells fluoresce red (i.e., ethidium bromide).
3.3.4.2.2. Oxygen Tension Levels and pH in Scaffold’s Interior Regions

The observed increasing cell survival trend can be attributed to the increased oxygen tension levels, as well as more normal pH levels in the interior of these scaffolds, as compared to that of control scaffolds after 21 day in vitro. With both, oxygen tension and pH levels, there was a positive correlation with the percentage of porogen used during fabrication (i.e., porosity) (Figure 3-15A). For instance, in respect to oxygen tension levels in the scaffold’s interior regions, the control scaffolds displayed the lowest oxygen tension levels (0.51% ± 0.47%), whereas 60% PLGA/40% NaCl scaffolds displayed the highest oxygen tension levels (4.11% ± 0.48%). However, there was no statistically significant difference in oxygen tension levels in the interior regions of scaffolds fabricated with 20% NaCl, 30% NaCl, or 40% NaCl. Although the oxygen tension levels in the interior regions of all scaffold groups (0%-40% NaCl) was significantly less than the oxygen tension levels in the media surrounding the scaffold (6.52% ± 0.61%).

Like oxygen tension levels, there was a positive relationship between the percentage of porogen used during fabrication and pH levels in the interior of the scaffolds (Figure 3-15B). pH levels are critical to cell survival, as too acidic or basic conditions is harmful to the cell. The media surrounding the construct (i.e., peri-cellular region) had a pH of 7.61 % ± 0.08%. Only scaffolds fabricated without NaCl (i.e., control scaffolds), and scaffolds with 10% NaCl displayed a significant difference in pH levels in the scaffold’s exterior regions compared to that in the exterior regions of the scaffold (i.e., pH gradient from scaffold’s exterior to interior regions) (i.e., 6.83%±0.06% and 6.99% ± 0.06%, respectively).
Figure 3-15. Comparison of oxygen tension and pH levels in interior of control and macro-porous PLGA microsphere scaffolds at 21 days in vitro.

3.4. Discussion

Large area or critically sized bone defect repair via scaffold-based bone tissue engineering requires a mechanically-stable scaffold that supports osteogenesis entirely. For this, it is critical to develop a scaffold that allows for oxygen diffusion, and thus, cell survival and proliferation in the scaffold’s interior regions. In the present study, we have developed a novel biodegradable scaffold for bone regeneration that encompasses the previously mentioned requirements, and demonstrated their ability to promote uniform osteogenesis in vitro.
Figure 3-16. The pros and cons of scaffolds with low and high porosity, and the requirement to design scaffolds with optimal porosity and mechanical properties (i.e., optimally porous scaffolds) for homogeneous and enhanced bone regeneration.

Appropriate scaffold porosity and accessible volume are critical for obtaining effective osteogenesis in large area bone repair. As seen in Figure 3-16, deviations from the moderate porosity range display positive and negative tradeoffs. Scaffolds with decreased average pore size are associated with an increase in surface area, and thus, cell seeding efficiency. Such scaffolds with relatively lower porosity exhibit higher mechanical strength, a critical factor in clinical applications (140). However, these scaffolds are also associated with significant drawbacks, including decreased mass transport of oxygen and nutrients, and decreased vascularization, which in turn, results in decreased osteoblast survival and bone regeneration (16, 17, 141, 142). On the other hand, scaffolds with high porosity are not as mechanically strong and display decreased cell seeding efficiency, but are associated with higher mass transport of oxygen and nutrients, facilitating enhanced bone regeneration (21). With respect to dynamic bone remodeling, increased osteoclast number and size in scaffolds with high porosity may
result in increased bone matrix strength via increases in bone remodeling (143). Also, studies have identified scaffolds with increased pore sizes to allow for the most efficient vascularization (78, 144). Thus, scaffold porosity is a crucial parameter to consider when fabricating scaffolds.

For bone tissue engineering applications, PLGA scaffolds developed via microsphere sintering techniques have a unique advantage as they display mechanical properties in the range of human cancellous bone (31), an essential aspect of scaffolds to ensure proper support at the defect site upon implantation. These cancellous bone-mechanically compatible scaffolds are attractive since bone has the special ability to undergo remodeling and optimize its mechanical function for its particular skeletal location, and thus, can be effectively used for regeneration in either cancellous or cortical bone sites (55). Our group has fabricated and extensively investigated PLGA 85/15, PLGA-nano hydroxyapatite composites, and PLGA-chitosan blend microsphere scaffolds for bone regeneration (31-33, 145). PLGA 85/15 based microsphere scaffolds have supported bone forming cell proliferation, differentiation and mineralization in vitro and bone formation in vivo (32). However, these PLGA microsphere scaffolds lack the necessary porosity for sufficient cell in-growth, and thus, result in surface-limited osteogenesis in vitro and in vivo (Figure 3-17).

Figure 3-17. Schematic illustration of surface-limited and large-area bone regeneration in a biodegradable scaffold. Scaffolds with a limited pore size and reduced oxygen diffusion through their pore structure result in bone cell survival
and growth limited to the scaffold surface, and thus surface-limited bone regeneration (B), while optimally porous scaffolds with increased oxygen levels in the scaffold interior allow for bone regeneration throughout the scaffold thickness, which, in turn, can support large-area bone regeneration both in vitro and in vivo (C). (A) is showing the cross-sectional surface of a scaffold.

To effectively increase microsphere scaffold porosity, we have used microsphere sintering followed by a porogen leaching method. In this method, we combined PLGA microspheres with a porogen (i.e., NaCl particles), thermally sintered, and then leached out the porogen by soaking the constructs in water. Scaffold porosity and mechanical properties can be tuned according to the clinical requirement by controlling the size and amount of the porogen added during the fabrication process. Through this method, we have improved PLGA microsphere performance and its ability to support osteoblast cell survival, proliferation and mineralization throughout the construct, and yet retained mechanical compatibility for effective bone regeneration.

As we increase the dry weight ratio of NaCl:PLGA used during the fabrication process of PLGA microsphere scaffolds, the porosity and accessible volume increases significantly (Table 3-). High accessible volume within scaffolds is a crucial parameter that influences the efficiency of nutrient, gas, and waste exchange within the scaffolds, as well as cell migration needed to promote tissue regeneration. Furthermore, it facilitates angiogenesis allowing for blood vessel in-growth, and thus, increases supply of oxygen and nutrients to the center of the construct. However, with increases in pore volume, mechanical integrity is sacrificed (Figure 3-6). Scaffolds with a higher ratio of NaCl than that in 40% NaCl/60% PLGA scaffolds were not mechanically stable. Scaffolds fabricated with an intermediate concentration of NaCl (i.e., optimally-porous scaffolds; 20% NaCl/80% PLGA scaffolds) were significantly more mechanically robust than those fabricated with 40% NaCl/60% PLGA.
In addition to decreasing mechanical strength, scaffolds with increased porosity display a lower cell seeding efficiency, as these scaffolds are less efficient in retaining cells during the cell seeding process (Figure 3-7). Despite the decrease in initial cell number seeded on the scaffolds with increased porosity, cell numbers on these scaffolds reached that of control scaffolds by 5 days in culture, and surpassed it by two weeks in culture (Figure 3-8). We demonstrated that scaffolds with increasing accessible pore volume corresponding specifically to pore sizes in the range of 200 to 400 µm (i.e., the critical pore size range for neovascularization of engineered bone constructs), resulted in
decreasing gradient of oxygen and pH from the exterior to interior regions of the MSC-seeded constructs after 21 days in vitro. For instance, scaffolds fabricated with 80% PLGA microspheres and 20% NaCl (by weight) demonstrate 30.6% of its accessible pore volume to pore sizes of 200 µm, 12.5% of its accessible pore volume to pore sizes of 300 µm, and 6.0% of its accessible pore volume to pore sizes of 400 µm, in contrast to 12.3%, 2.0% and 0.9% of that in scaffolds that did not undergo porogen leaching. Scaffolds fabricated with greater than 20% NaCl porogen further demonstrated increasing accessible pore volume, however significantly lower mechanical strength. Further, we demonstrated that increasing accessible pore volume corresponded to increasing oxygen tension and more normal pH levels in the interior of the scaffolds, allowing for enhanced MSC-derived osteoblasts survival throughout the construct after long-term 21 day culture. Thus, scaffolds with larger porosity have a better potential to support cell proliferation in vitro, likely due to uniform oxygen tension and near-neutral pH throughout the entire construct.

Increased cell survival and activity (i.e., osteopontin and Collagen Type 1 expression) was confirmed in the interior of scaffolds with increased porosity, compared to that of control scaffolds over a long-term culture. However, there appears to be an important relationship between mineralization potential and surface area of scaffold, since macro-porous scaffolds (i.e., 40% NaCl/60% PLGA scaffolds) did not display as high of mineralization as for optimally-porous scaffolds. Per these attributes, optimally-porous scaffolds display the highest performance in supporting cell infiltration, proliferation, and mineralization throughout the entire construct in vitro (Table 3-2).
Our optimally-porous scaffolds stand superior to other methods currently utilized to increase cell proliferation and mineralization throughout BTE constructs in vitro. For instance, bioreactor culture methods are popular alternative methods utilized to increase cell infiltration and proliferation throughout constructs (146-148). However, unlike bioreactor culture methods, which are complex in nature and only effective in vitro, optimally-porous scaffold development is simple and effectively allows for enhanced oxygen tensions throughout the constructs both in vitro and in vivo. In addition, the increased porosity in optimally-porous scaffolds are expected to improve vascularization and osteoclast participation, and hence, bone remodeling by closely mimicking the native bone repair process (143). Lastly, studies have cited a significant enhancement in bone regeneration when adding growth factors (i.e. BMP-2) to BTE constructs (149-151). However, functional bone regeneration may only occur when the entire construct, including the interior, supports cell survival and proliferation (i.e., fully osteoconductive). The combination of growth factors with an appropriate scaffold, such as our optimally-porous scaffold, that is fully osteoconductive and may support vascularization throughout, will lead to optimal bone regeneration in large area bone defects.

In this study, by controlling scaffold pore size and pore volume, we effectively designed oxygen tension controlled matrices. Increasing the amount of porogen resulted in a systematic increase in not only porosity, but also available oxygen tension throughout the matrix. The enhanced survival, proliferation, differentiation and mineralization of pre-osteoblasts may be attributed to the increase in available oxygen tension. However, this increased cell performance may be cell type specific, as other cell types, such as chondrocytes, display enhanced performance in scaffolds with low
porosity (152). Thus, the proposed optimally-porous scaffolds with improved oxygen availability and bone compatible mechanical properties are desirable for large area bone regeneration. Oxygen tension control via scaffold porosity optimization may provide opportunities in designing next generation scaffold systems most effective for large area/critical sized bone defect repair.

3.5. Conclusions

Large area or critically sized bone defects pose a serious challenge in orthopaedic surgery, as all current treatment options present with shortcomings. Bone tissue engineering offers a more promising alternative treatment strategy. However, this approach requires mechanically-stable scaffolds that support homogenous bone formation throughout the scaffold thickness. Despite advances in scaffold fabrication, current scaffold-based techniques are unable to support uniform, three-dimensional bone regeneration, and are limited to only the scaffold surface in vitro and in vivo. This is mainly due to inadequate scaffold pore sizes (<200 μm) and accessible pore volume, and the associated limited oxygen diffusion and vascular invasion. In this study, we have adopted a method combining microsphere sintering and porogen leaching techniques to fabricate scaffolds with increased accessible pore volume. Of the scaffolds developed, optimally-porous PLGA microsphere scaffolds were selected as most advantageous, since they retain mechanical strength in the range of human cancellous bone, and display significantly higher accessible pore volume, which is attributed to an increased percentage of larger pores (i.e., size range 200-600 μm). Unlike control scaffolds with limited pore size and accessible pore volume, optimally-porous scaffolds displayed
increased oxygen diffusion, pre-osteoblast cell infiltration, proliferation, and survival throughout the entire scaffold. Furthermore, optimally-porous PLGA microsphere scaffolds displayed enhanced and homogenous mineralization \textit{in vitro}. Since these newly designed optimally-porous scaffolds are weight-bearing, fully osteoconductive and have the ability to support vascularization, they may serve as effective scaffolds for large area bone defect repair/regeneration. In addition, this study demonstrates the ability to modulate scaffold porosity and in turn, develop oxygen tension controlled matrices that are effective for large area bone regeneration.
4. IDENTIFICATION OF EFFECTIVE AND CLINICALLY-RELEVANT PROGENITOR CELLS FOR ENHANCED VASCULAR AND BONE REGENERATION

4.1. Introduction

Insufficient neo-vascularization currently represents a critical roadblock to successfully engineering bone (35, 110, 142). The incorporation of endothelial cells into engineered bone constructs has been offered as a promising and efficient approach to enhance vascularization and in turn, promote successful bone formation at the graft site (40, 41, 106, 153). Though many previous pre-vascularization studies have used human umbilical vein endothelial cells (HUVECs) and mature endothelial cells obtained via sacrifice of blood vessels, they are not clinically applicable and have demonstrated significant apoptosis upon transplantation (154). Endothelial progenitor cells (EPCs), however, represent an exciting alternative to mature endothelial cells as they display high angiogenic, proliferative and survival potential in situ. Further, EPCs have been successfully isolated from a number of sources (i.e., peripheral blood, bone marrow, umbilical cord blood), and may be expanded to achieve adequate cell numbers for tissue engineering applications without losing their endothelial cell phenotype (122). Isolation of EPCs from peripheral blood eliminates donor site morbidity risks, and thus, may represent an ideal autologous cell source for the promotion of vascularization in tissue engineering applications.

In the present study, we have isolated EPCs from two clinically-relevant sources
for bone tissue engineering applications, peripheral blood and bone marrow (PB-EPCs and BM-EPCs, respectively). In attempt to identify a superior source for EPC isolation, we, for the first time, investigated the differential phenotypic expression of PB-EPCs and BM-EPCs, and their potential for angiogenesis in vitro. We also assessed their ability to enhance osteogenic differentiation of mesenchymal stem cells in vitro, and their enhancement of key angiogenic and osteogenic markers. This investigation presents a landmark study as it identifies the ideal isolation source for EPCs, making steps closer to achieving clinical success in bone regeneration and repair.

4.2. Materials and Methods

4.2.1. Isolation of Rabbit Mesenchymal Stem Cells (MSCs)

Mononuclear cells were isolated via layering over a Percoll density gradient, and centrifuging at 600 rpm for 20 min at room temperature. The mononuclear cell fraction was seeded and expanded in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% FBS and 1% Penicillin/Streptomycin (P/S, Gibco) at 37°C and 5% CO₂. Passages 3-5 were used for experimentation.
4.2.2. Isolation of Rabbit Endothelial Progenitor Cells (EPCs)

4.2.2.1. Bone Marrow-Derived Endothelial Progenitor Cells (BM-EPCs)

Mononuclear cells from the bone marrow of New Zealand White rabbits were isolated via layering over a Percoll density gradient, and centrifuging at 600 rpm for 20 min at room temperature. The mononuclear cell fraction was re-suspended in endothelial cell growth medium (EGM2, Lonza; composed of endothelial cell basal medium-2 (EBM-2), 10% FBS, 1% P/S and EGM-2-SingleQuots growth factors and supplements), immediately seeded on dishes coated with 1 mg/cm² of rabbit type I collagen (C5608, Sigma), and cultured at 37°C and 5% CO₂. Non-adherent cells were removed after 4-7 days with gentle washes of PBS. Culture medium was changed every three days. After 3 weeks of passaging and expanding on collagen-coated plates, approximately 30 million cells were achieved. Cells were cryopreserved for subsequent experiments. At this point, collagen coating was no longer required. Cells isolated 3-4 weeks post-isolation (passages 5-8) were used for experiments.

4.2.2.2. Peripheral Blood-Derived Endothelial Progenitor Cells (PB-EPCs)

Peripheral blood (50 mL) was collected via cardiac bleeding protocol (approved by the University of Connecticut Health Center Animal Care and Use Committee) from New Zealand White rabbits (Figure 4-1). Isolation of mononuclear cell fraction, and subsequent cell culture was performed following procedures described in Section 4.2.2.1.
Similar numbers of PB-EPCs (30 x 10⁶ total cells) were achieved after 3 weeks of culture, at which point PB-EPCs obtained a cobblestone-like morphology, and collagen coating the cell culture plates was no longer required. Cells isolated 3-4 weeks post-isolation (passages 5-8) were used for experiments.

![Figure 4-1](image)

**Figure 4-1.** (A) Isolation of rabbit peripheral blood via cardiac exsanguination (i.e., terminal bleeding). (B) Image of Percoll solution layered on top of peripheral blood in 50 milliliter centrifuge tubes prior to centrifugation. (c) Image of peripheral blood separated by Percoll density gradient after centrifugation. At this stage, the mononuclear fraction may be easily isolated.

4.2.3. **Isolation of Rabbit Vascular Smooth Muscle Cells**

Methods for the isolation of rabbit smooth muscle cells (SMCs) from the aorta were adapted from methods described by Sreejayan *et al.* (155). Passages 2-4 were used for experimentation.

4.2.4. **Culture of Human Umbilical Vein Endothelial Cells**

HUVECs (CRL-2873, ATTC) were cultured in EGM2 on 150 mm cell culture plates at 37°C and 5% CO₂.
4.2.5.  **Flow Cytometry**

Samples were incubated with the following primary antibodies anti-human CD31 (M0823), anti-human vWF (sc-59957), anti-human CD34 (sc-7045), anti-rabbit CD44 (MCA806G), and anti-rabbit CD45 (MCA808G), subsequently an appropriate fluorochrome-conjugated secondary antibody: mouse anti-goat IgG-PE (sc-3752) or goat anti-mouse IgG-PE (sc-3738). Samples were analyzed using a BD Biosciences LSR II using FACSDiVa software. PE was excited by the 488 nm laser and detected using a 575/25 bandpass filter. Live/Dead® Fixable Far Red Dead Cell Stain was excited by the 633 nm laser and detected using a 660/20 bandpass filter. Data was analyzed using FlowJo software v.8.7.3 (TreeStar).

4.2.6.  **Western Blot Analysis**

Western blot analysis was performed on cell lysates as described by Lo et al. using the following primary antibodies, alpha-smooth muscle actin (ab7817), transgelin (sc-18513), smoothelin (sc-20479) (156).

4.2.7.  **Matrigel 2D Assay**

Cell suspensions containing 150,000 cells in 150 µl of DMEM were seeded in triplicates on Matrigel coated glass bottom culture dishes, and plates were incubated for 6 hours at 37°C and 5% CO₂. Samples were then incubated with calcein-AM, and imaged
using confocal microscopy. For quantification, in ten random fields per sample, a pattern recognition values were determined following the manufacturer’s guidelines (ECM625, Millipore). Number of branch points and the number of tubular structures were counted under a phase contrast microscope. Results are expressed as mean ± standard deviation.

**4.2.8. Co-Culture of MSCs and EPCs**

Gene expression of osteogenic MSCs with PB-EPCs or BM-EPCs cultured simultaneously (i.e., co-culture) was examined. Prior to co-culture, MSCs were cultured in osteogenic media (DMEM, 10% FBS, 10 nM dexamethasone, 50 µg/ml ascorbic acid, 10 mM β-glycerophosphate) for 7 days. Then, MSCs were co-cultured with either PB-EPCs or BM-EPCs at various co-culture ratios in a 1-to-1 mixture of osteogenic and angiogenic medium on cell culture plates (not collagen coated). The following twelve sample groups were studied: PB-EPCs alone; BM-EPCs alone; MSCs:PB-EPCs co-culture ratios of 1:4, 1:2, 1:1, 2:1, and 4:1; and MSCs:BM-EPCs co-culture ratios of 1:4, 1:2, 1:1, 2:1, and 4:1. Specific test conditions for cellular expression studies are described in the following Section 2.10, 2.11 and 2.12.

**4.2.9. Analysis of Co-Culture Systems**

**4.2.9.1. Matrigel 3D Assay**

Cells were encapsulated in Matrigel (10⁶ cells/mL Matrigel) and cultured in a 1-to-1 mixture of osteogenic and angiogenic medium for 7 days in 48 well cell culture plates. The following twelve sample groups were studied: PB-EPCs alone; BM-EPCs
alone; MSCs:PB-EPCs co-culture ratios of 1:4, 1:2, 1:1, 2:1, and 4:1; and MSCs:BM-EPCs co-culture ratios of 1:4, 1:2, 1:1, 2:1, and 4:1. Three-dimensional samples (10 mm diameter, 2 mm height) were paraffin-embedded, sectioned and then stained with hematoxylin and eosin. Four samples per group were embedded, and three sections per sample were stained (one from top, middle and bottom; approximately 200 µm apart from each other). Stained sections were analyzed under the light microscope Olympus BX50 with Olympus DP70 camera. Twelve images viewed under 10X magnification, and the number of branches were counted for each sample.

4.2.9.2. **Alkaline Phosphatase Activity**

Alkaline Phosphatase (ALP) Substrate Kit (172-1063) was used in accordance to manufacturer’s instructions. ALP levels were normalized to total protein levels as determined using BCA Protein Assay Kit.

4.2.9.3. **Gene Expression (RT-PCR)**

For each cell culture condition (n=3), a total of 40,000 cells were plated on a 24 well-cell culture plate. For co-culture samples, 1 milliliter of a 1-to-1 mix of endothelial and osteogenic media was added. For single cell type cultures (i.e., EPCs or MSCs), 1 milliliter of endothelial or osteogenic media was added, respectively. At the appropriate time points, total RNA was isolated using the Qiagen RNeasy Mini Kit, and converted into cDNA via Clontech Sprint RT Complete cDNA synthesis kit. For quantitative real time PCR, BioRad iCycler Thermal Cycler Base and BioRad iQ Supermix, BMP-2
(Oc03824113\_s1), BMP4 (Oc03233792\_m1), thrombomodulin (Oc03822979\_s1), KDR/FLK (Oc03395666\_m1), collagen type 1 alpha 1 (Oc03396074\_g1), VEGF-A (Oc03395999\_m1), and GAPDH (Oc03823402\_g1) gene probes (Applied Biosciences, Carlsbad, CA) were used. Threshold cycle values of target genes was standardized against GAPDH expression and normalized to the expression in the control culture.

### 4.2.10. Statistical Analysis

For Matrigel 2D and 3D Assay analysis, ALP and gene expression quantification a two-way analysis of variance (ANOVA) was preformed to compare data. Error is reported in figures as the standard deviation (SD) and significance was determined using a probability value of P<0.050.
4.3. Results

4.3.1. Cell Morphology and Expression of Isolated BM-EPCs and PB-EPCs

Rabbit EPCs isolated either from bone marrow (BM) or peripheral blood (PB) by density gradient and cultured on collagen type 1-coated cell culture plates exhibited distinct morphology and cellular expressions of key endothelial cell markers (Figure 4-2). Initially, both PB-EPCs and BM-EPCs displayed a spindle-like morphology. However, after two to three weeks of culture post-isolation, PB-EPCs formed colonies that exhibited a pronounced endothelial “cobblestone” morphology, which was also observed...
by human umbilical vein endothelial cells (HUVECs), a commonly studied vascular endothelial cell line. BM-EPCs did not form colonies of cobblestone-like cells by this time, and continued to have colonies composed of spindle-shaped cells. HUVECs were used in these experiments to study the characteristics of a mature, homogenous vascular endothelial cell line.

Figure 4-3. Flow cytometry analysis of CD31, vWF, VEGF-R2, CD34, and CD45 on (A) HUVECs, (B) PB-EPCs, and (C) BM-EPCs; and CD44, CD34, and CD45 on (D) MSCs. Blue lines indicate fluorescence signals of isotypic controls, and red lines indicate fluorescence signals of the specific antigens. Plots are depicted with % of Maximum Counts (%Max) on y-axes, and the PE fluorescence intensity for the indicated markers is shown (bi-exponential scale) on x-axes.
Like HUVECs, PB-EPCs are strongly positive for CD31 and von Willebrand factor, common endothelial cell markers (Figure 4-3A, B). BM-EPCs, however, did not express CD31 (Figure 4-3C). All endothelial cell types (i.e., HUVECs, PB-EPCs, BM-EPCs) display a low signal for CD34 and CD45 (Figure 4-3). MSCs stained positive for CD44, and negative for CD34 and CD45 (Figure 4-3D). Lack of CD45 signal indicates no/minimal hematopoietic cell contamination.

![Gene Expression Chart](image)

**Figure 4-4.** Gene expression of key bone and vascular markers (i.e., BMP-2, COL I, BMP-4, VEGF, VEGF–R2 and thrombomodulin) in PB-EPCs and BM-EPCs 4 weeks post-isolation.

Furthermore, four weeks post-isolation, PB-EPCs also demonstrated markedly higher gene expression levels of key vascular markers important to angiogenesis and
osteogenesis. The following genes displayed significantly higher regulation in PB-EPCs in comparison to that of BM-EPCs, BMP-2, collagen type 1 (COL1), VEGF, BMP4, VEGF-receptor 2 (VEGF-R2/KDR), thrombomodulin (THRMB) (Figure 4-4). VEGF-R2 is a well-documented marker of EPCs, as they have been shown to respond to angiogenic factor, VEGF, for proliferation and migration. On the other hand, interestingly, BM-EPCs expressed high levels of established smooth muscle markers (i.e., smooth muscle actin- alpha (SMA-α), smoothelin, transgelin), whereas PB-EPCs did not (Figure 4-5C-E). MSCs expressed SMA-α, but lacked smoothelin and transgelin.

![Image](image_url)  

**Figure 4-5.** Protein expression of isolated cell populations.

### 4.3.2. **2D In Vitro Angiogenesis Characteristics of BM-EPCs and PB-EPCs**

PB-EPCs and BM-EPCs significantly differed in their angiogenic ability to form tubular networks. PB-EPCs, like HUVECs, uniformly participated in elaborate network
formations (Figure 4-6 A, B), whereas a significantly lower percentage of BM-EPCs
demonstrated network formation, and instead, the majority of BM-EPCs showed only the
ability to stretch on Matrigel (Figure 4-6 C, D). Also, PB-EPCs formed significantly
more branch points and higher total tube length than BM-EPCs; however, both form
significantly less than HUVECs (Figure 4-6 G).

Figure 4-6. Two-dimensional angiogenesis assay showing network formation by (A) HUVECs, (B) PB-EPCs and (C) BM-EPCs, (D) SMCs, and (E) MSCs. Scale bar = 500 µm. (E) Numerical pattern recognition values were assigned to each pattern, such that a numerical value is associated with a degree of angiogenesis progression (i.e., 0 = cells isolated or in a sheet-like monolayer, 1 = cells begin to migrate and align themselves, 2 = capillary tubes visible but no sprouting, 3 = sprouting of new capillary tubes visible, 4 = closed polygons form, 5 = complex mesh like structures develop). Examples of branch point (white arrow), tube length (white line), and polygon shape (white outlined hexagon) on indicated on (B). Comparison of number of (F) formed branch points, (G) average tube length and (H) total tube
4.3.3. 3D In Vitro Network Formation in Matrigel

After 1 week in 3D Matrigel culture, PB-EPCs began to organize into elongated structures that connected to each other and formed a 3D network (Figure 4-7A). In contrast, BM-EPCs did not form extensive 3D networks (Figure 4-7B, C). Co-seeding PB-EPCs and MSCs promoted the degree of network formation. Specifically, significantly more luminal structures were present in groups seeded with MSCs:PB-EPCs ratios of 1:4 compared to PB-EPC alone. A direct relationship was observed between branch points and PB-EPCs:MSCs co-culture ratio.

Figure 4-7. Capillary network formation of (A) PB-EPCs and (B) BM-EPCs in Matrigel. (C) PB-EPCs formed significantly higher branching points after 7 days cultured in Matrigel in vitro. Significance is signified as * (p<0.01). (D-I)
Hematoxylin/eosin staining of capillary network formation of various co-culture ratios of MSCs and PB-EPCs (MSCs:PB-EPCs), specifically (D) 1:4, (E) 1:2, (F) 1:1, (G) 2:1, (H) 4:1. (I) Scoring of the branch points formed with different MSC:EPC ratios in 3D culture, data presented as mean ± SD. Scale bar = 250 μm.

4.3.4. ALP Activity of BM-EPCs and PB-EPCs Co-Cultured with MSCs

Alkaline phosphatase (ALP) activity in co-cultures of MSCs with either PB-EPCs or BM-EPCs was studied as an early marker of osteogenic differentiation. After 14 days of culture, PB-EPCs displayed significantly higher ALP activity than BM-EPCs, though both less than that of MSCs (Figure 4-8). ALP activity levels were also enhanced in MSC:PB-EPC co-cultures, in comparison to MSCs or PB-EPCs cultured alone. ALP activity increased as the MSC fraction of the co-culture increased, however this trend was not observed in MSC:BM-EPC co-cultures.
Figure 4-8. Comparison of ALP activity of MSCs co-cultured with PB-EPCs or BM-EPCs at various ratios. ALP activity of MSCs, PB-EPCs, and BM-EPCs alone under the same conditions are displayed in dashed lines, whereas MSC:PB-EPC and MSC:BM-EPC co-cultures at different ratios are displayed as the blue and red line, respectively.

4.3.5. Effect of MSC:EPC Co-Culture Ratios on Vascular and Osteogenic Gene Expression

Gene expression of critical angiogenic and osteogenic markers are higher in PB-EPCs than BM-EPCs (dashed lines) after 7 days in vitro (Figure 4-9A-F). BMP-2 and BMP-4 expression levels display a direct relationship with the MSC fraction in the co-culture ratios of both MSC:PB-EPC and MSC:BM-EPC, such that BMP-2 and BMP-4 expression significantly increased as the MSC fraction in the co-culture increased (Figure 4-9A, B). For example, co-cultures with a 4:1 MSC:EPC ratio displayed the highest levels of BMP-2 and BMP-4. On the other hand, COL1 expression was also only enhanced in MSC:PB-EPC co-cultures, in comparison to MSCs and EPCs cultured alone; this trend was not observed in MSC:BM-EPC co-cultures (Figure 4-9C). Gene expression of vascular markers, VEGF, VEGF-R2 and thrombomodulin, decreased with increasing MSC fraction in both MSC:PB-EPC and MSC:BM-EPC co-culture ratios (Figure 4-9D-F).
Figure 4-9. Comparison of (A) BMP-2, (B) COL1, (C) BMP-4, (D) VEGF, (E) VEGF-R2, and (F) thrombomodulin in co-cultures with MSCs and either PB-EPCs or BM-EPCs at different ratios. Gene expression normalized to GAPDH levels is plotted on the y-axis, and various ratios of MSCs co-cultured with EPCs on the x-axis. Gene expression of MSCs, PB-EPCs, and BM-EPCs alone under the same conditions of the MSC:EPC co-cultures after 7 day in vitro are presented as mean and displayed in dashed lines, whereas MSC:PB-EPC co-cultures and MSC:BM-EPC co-cultures at different ratios are displayed as the blue and red line, respectively.

4.3.6. Time Course of BMP-2 and VEGF Expression of

MSC:PB-EPC Co-Cultures
Figure 4-10. Time course of (A) BMP-2 and (B) VEGF expression in co-cultures of MSCs and PB-EPCs at different ratios at day 2, 7, and 14 in vitro. Dashed lines indicate gene expression of either MSCs or PB-EPCs cultured alone under the same conditions as the co-cultures.

BMP-2 gene expression was significantly up-regulated in MSC:PB-EPC co-cultures over the course of 14 days in vitro (Figure 4-10A), in respect to BMP-2 expression in MSC alone. After 2 and 7 days in vitro, BMP-2 expression was enhanced in only 1:1, 2:1 and 4:1 MSC:PB-EPC co-culture ratios, in comparison to MSCs and PB-EPCs cultured alone. However, after 14 days, all MSC:PB-EPC co-culture ratios displayed significantly enhanced expression of BMP-2 to the expression of that in each cell type cultured alone.

VEGF gene regulation followed an inverse pattern of BMP-2 in MSC:PB-EPC co-culture. VEGF expression decreased as MSC fraction increased in MSC:PB-EPC co-culture, and was also significantly enhanced in co-culture compared to when cultured alone (Figure 4-10B). VEGF expression was enhanced in co-culture as early as day 2, with MSC:PB-EPC ratios of 1:1, 1:2 and 1:4 being significantly enhanced in comparison
to the cells alone. At day 7 and 14, VEGF expression was enhanced in all co-culture ratios in relation to its expression in the cells alone.

4.4. Discussion

Despite the recent exponential growth of EPC literature, critical details remain unclear. In this study, we determined the best clinically-relevant cell source for EPC isolation. Past studies investigated whether adult peripheral blood (PB) or fetal cord blood (CB) offer the best source for EPC isolation. Many reported that CB represent a superior EPC cell source, as CB-EPCs had greater isolation efficiency, higher ex vivo cell number expression, and longer stability of vascular networks in vivo, in comparison to PB-EPCs (104). Though interesting, CB-ECPs are presently not clinically relevant, as most patients do not have access to this. With this, we sought to explore not only more clinically-relevant cell sources, but also investigate more widely reported sources in bone tissue engineering studies. We have isolated, characterized and compared EPCs from rabbit peripheral blood and bone marrow (PB-EPCs and BM-EPCs, respectively). Though the investigated cells were isolated from rabbits, the same cell populations may be isolated from humans for their potential clinical use for bone repair and regeneration (104, 157). PB-EPCs have proven to be a superior endothelial cell population, in contrast to BM-EPCs, a population in which the majority of bone tissue engineering studies involving EPCs have previously reported on.

Differences in isolation and cultivation procedures of EPCs make it difficult to directly compare published studies on the outcomes of EPC functionality. For this reason, we have isolated and cultured EPCs isolated from peripheral blood and bone marrow in a
similar manner, and as previously described by numerous studies (158). The PB-EPC population that has been characterized in our studies has also been referred to as late EPCs, outgrowth endothelial cells (OECs) and endothelial colony–forming cells (ECFCs) in other published studies (158-160). BM-EPCs did not express endothelial marker CD31, as confirmed by flow cytometry and western blot analysis.

Bone formation is a complex process, and one of the most important heterotypic cross-talks in this process is the one between endothelial cells and osteoblasts (39, 161). The underlying cellular regulatory mechanisms for this cross-talk have pointed to paracrine signaling based on angiogenic growth factors, such as VEGF and PDGF, and also direct cellular communication via gap junctions (162). Therefore, we also investigated how the addition of isolated EPCs contributes to osteogenic differentiation and bone-forming capacity of rabbit mesenchymal stem cells (MSCs) for bone tissue engineering purposes, a popular approach actively being investigated in vivo by many other groups (40, 42, 47, 153). In co-culture, rabbit MSCs and PB-EPCs display significantly enhanced expression of key vascular and osteogenic markers. The expression of the key markers were also significantly higher in MSC/PB-EPC co-culture than that in co-cultures of MSCs and BM-EPCs. As early as 1 week in co-culture, expression of bone markers COL1, BMP-2, and BMP-4, and angiogenic markers VEGF, VEGF-R2, and thrombomodulin were enhanced beyond expression levels of MSCs and PB-EPCs cultured alone.
Figure 4-11. Schematic illustration of possible EPC differentiation mechanism.

The putative BM-EPCs, which were isolated precisely as many tissue engineering researchers have previously described, did not exhibit the typical endothelial phenotype as expected of EPCs. Instead, putative BM-EPCs exhibited more of a smooth muscle-like phenotype, with limited angiogenic potential similar to the smooth muscle cells isolated from rabbit aorta, as compared to HUVECs and PB-EPCs. Further, putative BM-EPCs express high levels of smooth muscle markers, whereas PB-EPCs do not. Plausible explanations for these observations involve the committed lineage stages of BM-EPCs and PB-EPCs isolated from bone marrow and peripheral blood, as illustrated in Figure 4-11. It has been established that EPCs originate from the bone marrow, and are derived from a vascular progenitor cell population, which also has the ability to give rise to smooth muscle progenitor cells (SMPCs) (163). As EPCs become mobilized from the bone marrow into circulation in the peripheral blood, they begin to express more
endothelial cell markers, and begin to lose their stem cell-like properties and become less plastic. Thus, the transition from the bone marrow to the peripheral blood marks a certain degree of maturation or committed-ness of EPCs to the endothelial cell lineage. EPCs and SMPCs have previously been shown to have the ability to trans-differentiate from one lineage to the other via an endothelial-to-mesenchymal transition-like process mediated by TGFβ-RI (164). Thus, BM-EPCs, which may harbor more stem cell-like properties and more easily trans-differentiate into SMPCs, are marked by the waning of endothelial markers and functionality. Whereas, PB-EPCs are likely to be in a more committed stage in their differentiation process, and do not give rise to smooth muscle progeny spontaneously.

Supporting our study, Fedrovich et al. observed early BM-EPCs (i.e., BM-EPCs cultured for approximately 1 week in endothelial growth media) to express CD31 only transiently, and lose CD31 expression as culture time increased (43). BM-EPCs also displayed transient endothelial cell phenotype, including isolectin B4 binding and acetylated LDL-incorporation. Also, it is important to note that in other previous studies that report their isolated BM-EPCs to be CD31 positive, they may have studied their BM-EPCs at this early stage. Also, many other studies have not specified whether their BM-EPCs express CD31, they only provide their isolation protocol.

These putative BM-EPC may possibly be more appropriately referred to as smooth-muscle like progenitor cells; however, although not a superior endothelial progenitor cell source, this population may still serve as an important option for enhanced vascularization and/or bone formation in engineered bone tissue. Firstly, there is an established cooperation between endothelial cells and perivascular cells (i.e., smooth
muscle cells located at the periphery of capillaries) that is fundamental for vascular maturation (35). Also, perivascular cells have been shown to differentiate into osteoblasts, and contribute to bone formation in vivo (165). Lastly, bone tissue engineering studies have reported positive results when implanting not only PB-EPCs, but also BM-EPCs, at a bone defect site, for enhanced vascularization and bone formation in vivo. Thus, given these established relationships between EPCs, perivascular cells and MSC derived-osteoblasts, future studies should upgrade co-culture systems, and perhaps explore tri-cultures involving these three cell types (i.e., PB-EPCs, putative BM-EPCs and MSC derived-osteoblasts) for further enhancement of bone formation and vascular networks in vivo.

4.5. Conclusions

For tissue engineering applications, effective bone regeneration requires rapid neo-vascularization of implanted grafts to ensure the survival of cells in the early post-implantation phase. Incorporation of autologous endothelial progenitor cells (EPCs) for the promotion of primitive vascular network formation ex vivo has offered great promise for improved graft survival, enhanced rate of vascularization and bone regeneration in vivo. For clinical usage, identification of an optimal EPC isolation source from the patient is critical. We have, for the first time, characterized and directly compared EPCs from rabbit peripheral blood and bone marrow (PB-EPCs and BM-EPCs, respectively). PB-EPCs outperformed BM-EPCs on all measures. PB-EPCs displayed typical endothelial cell markers, such as CD31, as well as high angiogenic potential in three-
dimensional extracellular matrix \textit{in vitro}. Furthermore, PB-EPCs cultured simultaneously with mesenchymal stem cells, displayed significantly enhanced expression levels of key osteogenic and vascular markers, including alkaline phosphatase, bone morphogenetic protein 2, and vascular endothelial growth factor. On the contrary, putative BM-EPCs did not express CD31, and instead, expressed key smooth muscle markers. BM-EPCs further failed to display vasculogenic activity. Hence, the highly angiogenic peripheral blood derived-EPCs may serve as an ideal cell population for enhanced vascularization and success of engineered bone tissue.
5. EVALUATION OF PRE-VASCULARIZED OPTIMALLY-POROUS SCAFFOLDS FOR ENHANCED NEO-VASCULARIZATION AND BONE FORMATION IN VIVO

5.1. Introduction

Many cellular-based bone tissue engineering approaches involve the utilization of bone marrow derived mesenchymal stem cells (MSCs) as osteo-progenitor cells to promote and accelerate bone regeneration. In this approach, MSCs are seeded on scaffolds for colonization and implantation at a defect site. However, upon implantation, colonized cells in the interior regions of scaffolds with depth dimensions greater than one-millimeter experience limited oxygen and nutrient availability, since they are dependent on post-implantation vascularization that may occur on the order of days to weeks. Due to limited nutrient delivery and waste products removal via diffusion and proper functional vascularization, cell viability, and in turn, bone regeneration and host integration is severely hindered.

Efficient methods to establish near-immediate neovascularization in bone tissue engineered constructs are essential. In vitro pre-vascularization of bone tissue engineering constructs with endothelial progenitor cells (EPCs) have gained significant attention in this respect (48). EPCs, which may be easily isolated from the patient’s peripheral blood, demonstrate high proliferative potential for ex vivo expansion, as well as the ability to enhance and accelerate neovascularization in vivo (166). Moreover,
when co-implanted with MSCs at a bone defect site enhanced neovascularization and bone formation is observed. This enhancement is a result of synergistic communication (i.e., “cross-talk”) between MSCs and EPCs (167, 168), where MSCs have been shown to release angiogenic factor VEGF (169, 170), and EPCs have been shown to release osteogenic factor, such as BMP-2 and BMP-4 (171, 172). Although this pre-vascularization approach with EPCs and MSCs has been examined and proven effective, the ideal cell ratio that would potentially yield functional bone grafts has not yet been investigated.

In this study, we investigated a two-pronged approach for enhanced neovascularization and bone formation in vivo. We utilized our optimally-porous scaffolds with tunable, increased porosity, especially in the range of 300-400 um, which has been cited as the critical pore size range for neo-vascularization (16). These scaffolds demonstrate enhanced performance, specifically in respect to increased oxygen tension levels, near-normal pH levels, and increased cell viability in the interior regions of the constructs after long-term in vitro culture with MSCs. Moreover, we investigated the effectiveness of various co-culture ratios of MSCs and EPCs on these scaffolds in promoting neo-vascularization and bone formation in vivo.

5.2. Materials and Methods

5.2.1. In Vitro Culture of Cell-Seeded Optimally-Porous Scaffolds
Optimally porous scaffolds (20% NaCl/80% PLGA scaffolds; 10 mm diameter, 2 mm height) were seeded with a total of 250,000 cells/scaffold and cultured in a 1-to-1 mix of endothelial and osteogenic media for 2 days 37°C and 5% CO₂. Prior to culture on scaffolds, MSCs were cultured in osteogenic media (DMEM, 10% FBS, 10 nM dexamethasone, 50 µg/ml ascorbic acid, 5 mM β-glycerophosphate) on tissue culture plate (TCP) for 7 days. Various cell conditions seeded on scaffolds were examined: (1) MSC-derived osteoblasts, (2) EPCs, (3) co-culture of 4 parts MSC-derived osteoblasts to 1 part EPC (4:1), (4) co-culture of 1 part MSC-derived osteoblasts to 1 part EPC (1:1), and (5) co-culture of 1 part MSC-derived osteoblasts to 4 parts EPC (1:4).

5.2.2. In Vitro Performance Evaluation of Cell-Seeded Optimally-Porous Scaffolds

Expression of other osteogenic markers, RunX2 and Collagen Type I, and vasculogenic markers, CD31 and vWF, was analyzed by immunoﬂuorescence. After 2 days in vitro, cell-seeded constructs were fixed in 10% formalin for 1 hour at room temperature, rinsed with PBS, and permeabilized with 0.25% Triton X-100 for 10 minutes. Constructs were rinsed with PBS, blocked in 10% normal goat serum for 1 hour, and then incubated with the following primary antibodies for 1 hour: anti-RunX2 antibody (Abcam, ab76956, 1:50), anti-Collagen I antibody (Abcam, ab34710, 1:100), anti-CD31 antibody (Abcam, ab28364, 1:50), anti-Von Willebrand Factor antibody (Abcam, ab6994, 1:1000), Anti-β-Tubulin (Millipore 05-661, 1:200). Samples were then washed and labeled with the corresponding secondary antibody: anti-mouse IgG secondary antibody-FITC (sc-2099, Santa Cruz) or anti-rabbit IgG secondary antibody-
FITC (sc-53805, Santa Cruz) diluted 1:100 in 1% BSA/PBS for 40 min at room temperature. Finally, cell nuclei were counterstained using propidium iodide (81845, Sigma, St. Louis, MO). Stained constructs were examined via confocal microscopy (Zeiss LSM Confocor 2).

5.2.3. In Vivo Performance Evaluation of Cell-Seeded Optimally-Porous Scaffolds

First, to evaluate the most effect ratio of mesenchymal stem cells (MSCs) and peripheral-blood derived endothelial progenitor cells (PB-EPCs) seeded on our optimally-porous scaffolds for the promotion of vascularization in vivo, we utilized a SCID mouse subcutaneous implant model. Second, we evaluated the performance in respect to vascularization and bone regeneration potential of our optimally-porous scaffolds seeded with the most appropriate cell progenitor ratio and implanted in a rabbit ulnar bone defect model (Figure 5-1).

Figure 5-1. Study plan for evaluating the vascular and bone regeneration potential of optimally-porous scaffolds seeded with clinically-relevant progenitor cells in vivo.
5.2.3.1. **SCID Mouse Subcutaneous Implant Model**

5.2.3.1.1. Construct Implantation

To evaluate the *in vivo* performance of various combination of peripheral blood (PB)-derived EPCs (PB-EPCs) and MSC-derived osteoblasts, we implanted the 5 construct conditions described in Section 5.2.1 after 2 days *in vitro* to ensure proper cell adherence (Figure 5-2). As a negative controls, we used an acellular optimally-porous scaffolds. Male Fox Chase (CB17) SCID (severe combined immunodeficiency) (Charles River Laboratories, Wilmington, MA, USA) mice were anaesthetized with isoflurane in the induction chamber at a range of 3-5%. The animals were maintained on 2-4% isoflurane with the nose cone between placement of implants in separate subcutaneous dorsal pockets (2 implants/ animal; 3 animals/condition). The animals were post-operatively treated with the analgesic buprenorphine (0.05mg/kg, subcutaneous). All animal experiments were approved by the University of Connecticut Health Center Animal Care and Use Committee (protocol 2009-593).
Figure 5-2. Implantation of constructs in a dorsal subcutaneous SCID mouse model. (A, B) An incision with approximately the same length as the construct’s diameter is created in the dorsum of the mouse, (C) the construct is implanted subcutaneously, and (D) the skin at the implantation site is stapled closed.

5.2.3.1.2. Histological Staining and Analysis

The implanted constructs were retrieved 8 weeks after implantation to analyze the blood vessel formation and bone formation. Samples were fixed overnight in 10% formalin and processed for paraffin sections. Histological staining was performed with Masson’s Trichrome Staining (Sigma, HT15) on 5 µm thick paraffin sections according to the manufacturer’s protocol. Stained sections were analyzed under the light microscope Olympus BX50 with Olympus DP70 camera. For collagen quantification,
we utilized the RBG plug-in in ImageJ (National Institutes of Health), and specifically, collagen, which stains blue via Masson’s Trichrom Staining, was quantified. Ten random images under 40X magnification were analyzed per sample (3 samples/group; thirty total images per group) in ImageJ using the RBG Measure Plug-in (http://rsb.info.nih.gov/ij/plugins/color-histogram.html) to measure the Collagen stained blue with Trichrome Stain. Images were opened in Image J and converted to RBG Color (Image > Type > RBG Color), then the background was subtracted (Process > Subtract Background > Light Background), the RBG was measured on the selected region of interest (ROI). For vessel quantification, we analyzed and counted vascular structures in ten random images taken under 20X magnification for each group.

5.2.3.1.3. Immunohistochemical Analysis

To confirm vascularization and bone formation, we performed immunohistochemical analysis for endothelial markers CD31 and vWF, and bone markers RunX2 and Collagen Type I, respectively. We immune-stained sections of the group containing the scaffold seeded with 1 part MSC-derived osteoblasts and 1 part EPC (1:1). We used paraffin-embedded mouse limbs (post-natal day 1) as a positive control. Briefly, rehydrated sections were exposed to heat-mediated antigen target retrieval (Target Retrieval Solution, Dako S1700) for 5 minutes at 98°C, then blocked in 10% normal goat serum in PBS for 1 hour at room temperature. Sections were then incubated with the following primary antibodies overnight at 4°C: anti-RunX2 antibody (Abcam, ab76956, 1:50), anti-Collagen I antibody (Abcam, ab34710, 1:100), anti-CD31 antibody (Abcam, ab28364, 1:50), anti-Von Willebrand Factor antibody (Abcam, ab6994, 1:1000),
Anti-β-Tubulin (Millipore 05-661, 1:200). Samples were then washed and labeled with the corresponding secondary antibody: anti-mouse IgG secondary antibody-FITC (sc-2099, Santa Cruz) or anti-rabbit IgG secondary antibody-FITC (sc-53805, Santa Cruz) diluted 1:100 in 1% BSA/PBS for 40 min at room temperature. Finally, sections were mounted with Propidium Iodide/Anti-fade Solution (S7112, Millipore), and examined via confocal microscopy (Zeiss LSM ConfoCor2, 20X magnification).

5.2.3.2. **Rabbit Ulnar Defect Model**

5.2.3.2.1. Construct Implantation

Optimally porous scaffolds (20% NaCl/80% PLGA scaffolds; 15 mm height, 5 mm diameter) were seeded with a total of 500,000 cells/scaffold and cultured in a 1-to-1 mix of endothelial and osteogenic media for 2 days 37°C and 5% CO₂. Prior to culture on scaffolds, MSCs were cultured in osteogenic media (DMEM, 10% FBS, 10 nM dexamethasone, 50 µg/ml ascorbic acid, 5 mM β-glycerophosphate) on tissue culture plate (TCP) for 7 days. Various cell conditions seeded on scaffolds were examined: (1) MSC-derived osteoblasts, (2) EPCs, (3) co-culture of 1 part MSC-derived osteoblasts to 1 part EPC (1:1), (4) acellular (i.e., scaffold alone).

New Zealand white rabbits (4-5 kg weight) were anesthetized via an intramuscular injection of a mixture of ketamine (50mg/kg), xylazine (6mg/kg), and acepromazine (1mg/kg) (Figure 5-3). The right forelimb of the rabbit were shaved, and prepped with betadine and 70% ethanol. A longitudinal incision was made to expose the mid-diaphysis of ulna. A segment of the ulna measuring 15 millimeters in length was
removed using a bone saw. The scaffolds were implanted into the defect site and the
wound was closed by suturing muscles and skin in layers (Figure 5-4). After 12 weeks
post-implantation, animals were sacrificed and ulnar bone was excised, fixed in 10%
formalin for 24 hours, and then transferred to 70% ethanol until further analysis.

Figure 5-3. Preparation for rabbit ulnar bone defect model (A) Instruments
utilized during implantation of constructs in a critical-size ulnar bone defect in a
rabbit. The rabbit is prepared pre-surgery. The rabbit is (B) intubated, (C) hooked
up to a heart monitor, and (D) closely monitored during the operation. (F) The
rabbit’s right arm is prepared, cleansed with betadine and ethanol and stabilized.
Figure 5-4. Steps involved in implantation of a construct into a rabbit ulnar bone defect model. (A) An incision is made in the right arm of the rabbit, (B, C) the surrounding muscle is dissected to expose the ulnar bone. (D) A saw is used to make the first cut into the bone. (E) The length of the construct is marked off on the back of a sterile wooden stick, and (F, G) is used to measure the length of the bone defect. (H) A second cut is made in the bone, and the 15 millimeter bone piece is removed. (I) The bone and construct are equivalent in length. (J) The construct is secured in the defect, and (K) the surrounding muscle layer is sutured, and (L) then the surrounding skin is sutured.

5.2.3.2.2. X-Ray Analysis

Bone formation on each rabbit ulnar explant was analyzed at 18 kV for 10 seconds using the Faxitron X-ray machine, and the Faxitron DX-Beta SR v1.4 software.

5.2.3.2.3. MicroCT Analysis

MicroCT provides an efficient method to measure the distribution and density of
mineralized tissue throughout the scaffold. Limbs harvested at week 12 were imaged using cone beam micro-focus X-ray computed tomography to render three-dimensional models for direct quantitation of sample bone density and volume, and to provide a three-dimensional reconstruction of the defect (µCT40, Scanco Medical AG, Bassersdorf, Switzerland). Segmentation of bone and scaffold provided direct volumetric quantification of bone formation into the depths of the scaffold, as well as the analysis of the structural integrity of new bone and biodegradable scaffold. Mass was calibrated to a stepped hydroxyapatite phantom (Scanco, Item # KP-03-03) units of mg of HA per cubic cm (mg HA/cm³) (Figure 5-5).

![Figure 5-5. Image of the stepped hydroxyapatite phantom used for calibration of mass during MicroCT analysis.](image)

Serial tomographic images were acquired at 55 kV and 145 µA with a 300 msec integration time. A set length of 11 millimeters was analyzed within the defect.
Measurements include volumetric basis, but accurate measures are based on mass, which captures all mineral including what is not taken into account in volume measurements of repairing bone with low density regions. Mass is calibrated to a stepped hydroxyapatite phantom, units of mg of HA per cubic cm (mg HA/cm$^3$). Since the bone remodeling is significant, such that formation/resorption/remodeling are not distinguishable and includes regions of high and low density, to measure all formation, the total mass of all bone (radius, callus, everything) was measured within the set length. We also measured and subtracted off the mass of the radius in the intact limb within the matching region of each experimental limb.

5.2.3.2.4. Histological Staining and Analysis

Limbs were embedded in methyl methacrylate using a slow methylmethacrylate (sMMA) processing, infiltration and embedding techniques as described by Kecena et al., and then sectioned at 7 µm thickness with a Reichert Jung Polycut E microtome and a Tungsten carbide D profile knife (Dornt Hart), and mounted onto glass slides. These sections were then be stained with hematoxylin and eosin to evaluate cellular events, von Kossa to evaluate all the mineralized tissue at the site, and with Goldner’s Trichrome to evaluate the osteoid, or new unmineralized bone being deposited at bone forming sites. All staining was performed according to protocols described by Kecena et al. (173).

5.2.4. Statistical Analysis

For vascular and bone quantification analysis (i.e., histology and MicroCT), a two-way analysis of variance (ANOVA) was preformed to compare data. Error is
reported in figures as the standard deviation (SD) and significance was determined using a probability value of $p < 0.05$.

5.3. Results

5.3.1. Cell-Seeded Constructs In Vitro Evaluation

Cell-seeded macro-porous scaffolds were evaluated after 2 days in culture (i.e., pre-implantation stage) (Figure 5-6). Each macro-porous scaffolds was seeded with a total of 250,000 cells, and the following cellular conditions were examined in vitro to confirm cell performance, phenotype and survival at the pre-implantation stage: (1) MSC-derived osteoblasts, (2) EPCs-, and (3) co-culture of MSC-derived osteoblasts and EPCs at a 1:1 ratio. As seen in Figure 3, MSC-derived osteoblasts maintained their differentiation as demonstrated by positive immune-staining of bone markers collagen type 1 (Col1) and RunX2, which was observed in MSC-derived osteoblast – seeded constructs, as well as co-culture seeded constructs. Furthermore, EPCs maintained the endothelial cell phenotype as demonstrated by the positive immune-staining of vascular endothelial markers CD31 and von Willebrand Factor (vWF), which was observed in EPC – seeded constructs, as well as co-culture seeded constructs.
5.3.2.1. **SCID Mouse Subcutaneous Implantation**

Two day pre-cultured *in vitro* constructs were implanted for 8 weeks subcutaneously in SCID mice, at which point they were harvested for histological
analysis. Constructs of various MSC-derived osteoblast: EPC (OB:EPC) co-culture ratios were evaluated, and constructs of each cell type seeded alone served as controls, as well as acellular constructs. Constructs were paraffin-embedded, sectioned and processed for immunostaining and Masson’s Trichrome staining (Figure 5-7).

![Figure 5-7. Masson’s Trichrome Staining on (A) acellular, (B) MSC-seeded, (C) EPC-seeded, (D) 4 MSC: 1 EPC – seeded, (E) 1 MSC: 1 EPC –seeded, and (F) 1 MSC: 4 EPC- seeded constructs 8 weeks post-implantation.](image)

We analyzed and quantified collagen formation histologically (Figure 5-8B). Specifically, we performed a colorimetric quantification analysis (RBG plugin, ImageJ, NIH) on Masson’s Trichrome stained paraffin-embedded sections of the constructs. Since the Masson’s Trichrome staining procedure results in blue-stained collagen and bone, we utilized the RBG Measure Plug-in tool in Image J to measure the blue-staining collagen/bone. In this manner, we observed significant collagen formation in constructs cultured with MSC-derived osteoblasts, as well as with co-cultured MSC-derived osteoblasts and EPCs at all examined ratios (i.e., 4:1, 1:1, 1:4) compared to acellular
constructs after 8 weeks in vivo. However, in comparison to collagen formation in constructs seeded with MSC-derived osteoblasts, only constructs seeded with co-cultured MSC-derived osteoblasts and EPCs at ratios of 4:1 and 1:1 demonstrated significant collagen formation. On the other hand, subcutaneous implantation of acellular and EPC-seeded constructs resulted significant adipose tissue formation, instead of collagen formation.

Vascular formation analysis throughout the constructs was also performed on the Trichrome-stained sections. Implantation of EPCs significantly enhanced vascularization throughout the constructs. As seen in Figure 5-8, constructs seeded with EPCs demonstrated significantly more vascularization than constructs that were initially implanted without cells. However, constructs co-seeded with MSC-derived osteoblasts and EPCs at ratio of 1:1 (1 OB: 1 EPC) demonstrated the highest level of vascularization. Implantation of constructs co-seeded with MSC-derived osteoblasts and EPCs at ratio of 1:4 did result in significant vascularization compared to acellular constructs, however lower than that of EPC-seeded and 1:1 (1 OB: 1 EPC)–seeded constructs. It is important to note that significant adipose tissue formation was observed in implanted acellular constructs and constructs seeded with EPCs or higher level of EPCs in co-culture (1 OB: 4 EPC). Qualitative assessment of vascular, collagen and adipose tissue formation is shown in Figure 8. Thus, 1 OB: 1 EPC constructs showed the highest level of vascularization and collagen formation. Vessel formation was confirmed via CD31 and von Willabrand Factor (vWF) immunostaining, and bone formation was confirmed via Collagen type 1 (Col1) and RunX2 immunostaining (Figure 5-9).
Figure 5-8. (A) Quantification of In Vivo Vascularization. (** Significance compared to Negative (p < 0.05), # Significance compared to EPC (p < 0.05)). (B) Quantification of collagen staining. (** Significance compared to Negative (p < 0.05); # Significance compared to MSC (p < 0.05)).

Figure 5-9. Immunofluorescence staining of endothelial markers CD31 and vWF, and osteogenic markers.
RunX2 and Collagen type I on (A) construct pre-seeded with MSC:EPC (1:1), and (B) mouse limb post-natal day 1 (positive control).

5.3.2.2. **Rabbit Ulnar Critical Size Bone Defect Implantation**

A 15 millimeter bone defect was created in the ulnar bone of New Zealand White rabbits (4-5 kilograms), and an optimally-porous construct (cylinder of 5 millimeter diameter, and 15 millimeter height) was inserted into the bone defect. The construct was previously seeded with 250,000 mesenchymal stem cells (MSCs), endothelial progenitor cells (EPCs), or a 1-to-1 ratio of MSCs and EPCs (125,000 MSCs and 125,000 EPCs), or no cells (i.e., acellular construct to serve as a negative control), and cultured for 2 day in a 1-to-1 mix of osteogenic and endothelial growth media. We examined 6 rabbits for each group (i.e., 4 groups; acellular, MSC, EPC, 1 MSC:1 EPC). As seen in Figure 5-10, x-ray analysis was utilized immediately post-operation to confirm a bone defect of 15 millimeters was created.
Figure 5-10. Radiograph of rabbit (A) sham (i.e., no defect) arm, and (B) right arm (with defect) immediately post-implantation.

Rabbits were monitored closely for their recovery and performance. Several days to one week post operation, all rabbits were sitting up, walking, eating, drinking, and performing as expected for post-operation. Rabbits were fully functioning in their movements (i.e., walking, running, jumping), and applying weight on their forearms (including their arm that operated on) at 6 and 12 weeks post-operation. After 12 weeks, all rabbits were sacrificed for analysis and evaluation of bone and vascular regeneration at the bone defect.
MicroCT analysis was utilized to evaluate bone regeneration at the defect site. As seen in Figure 5-11, significant remodeling and callus formation was observed in most bone specimens, such that formation, resorption, and/or remodeling are not distinguishable, and includes regions of high and low density. Therefore, in order to measure all bone formation, the total mass of all bone (i.e., ulnar, radius, callus) within the set length was measured. In addition, the mass of the radius in the intact limb also measured. To quantify the mass and bone volume within the ulnar defect, the mass of the intact radius was subtracted off the mass of the matching region of each experimental limb.

Limited bone regeneration was observed in the acellular group, which confirmed this model as a critical-sized bone defect (Figure 5-12). The MSC and EPC group displayed higher levels of bone regeneration than the acellular group (Figure 5-13).
However, the rabbit group that received constructs seeded with 1-to-1 ratio of MSCs and EPCs displayed significantly higher levels of bone regeneration than that of the acellular group. As seen by the longitudinal sections in Figure 5-12, the 1 MSC: 1 EPC group displayed complete bone bridging throughout the thickness of the defect, whereas the other groups did not. Furthermore, as seen in Figure 5-14, the highest amount of regenerated bone volume and mass was observed in the 1:1 MSC:EPC group.

![Figure 5-12](image)

Figure 5-12. MicroCT analysis of ulnar bone defect 12 weeks post-implantation. Representative scout view images (i.e., radiographs), three-dimensional reconstructed images, and longitudinal section images are shown for each group (i.e., Acellular, MSC-seeded, EPC-seeded, and a 1:1 ratio of MSC:EPC seeded construct). Scout views were utilized to determine the region of interest (ROI) for bone mass and volume quantification. Scale bar = 5 millimeters.
Figure 5-13. (A) Bone Volume and (B) Mass with the ulnar bone defect. * indicates p < 0.05.

Figure 5-14. Representative histological images of Von Kossa-stained cross-sections (top two rows) and longitudinal sections (bottom two rows). Images were taken under low magnification to view the entire defect area in the section. Images displayed under 4X represent regions of interest (highlighted in red box) under low magnification. Scale bar (low magnification) = 2 millimeters. Scale bar (4X magnification) = 500 µm.
Histological analysis of Von Kossa stained sections confirmed the findings from MicroCT analysis (Figure 5-14). The acellular group displayed a lack of mineralized tissue within the defect, whereas the cell-seeded groups with MSCs and EPCs showed increased bone mineralization located at the bony edges of the bone defect. Moreover, the 1:1 MSC:EPC–seeded construct group displayed the highest amount of the bone mineralization not just localized to the bony edge of the defect, and instead there are signs of attempted bony bridging. Quantitative analysis of the Von Kossa stained sections demonstrated a significant increase in mineralized bone area in MSC- and EPC-seeded groups compared to acellular constructs, however their levels are not significantly different from each other. The co-culture 1:1 MSC:EPC-seeded group displayed the most significant increase in mineralized bone area compared to the acellular group (Figure 5-14, Figure 5-15).
Figure 5-15. Quantification of mineralized bone area formed within the rabbit ulnar defect. Three regions on Von Kossa stained sections were analyzed from 3 samples in each group under low magnification with a thresholding analysis tool from ImageJ software. ** indicates $p < 0.05$, ## indicates $p < 0.001$.

Similar trends were observed with unmineralized bone formation with Goldner’s Trichrome staining (Figure 5-16, Figure 5-17). Green staining represents collagen deposition, with the increased intensity being mineralized collagen formation. Red staining represents fibrous tissue formation. The acellular group showed limited collagen deposition, and increased levels of adipose tissue deposition (Figure 5-16). The MSC- and EPC- seeded groups displayed mineralized bone formation within the defect, as well as some unmineralized bone formation. However, the 1:1 MSC:EPC- seeded group displayed the highest levels of mineralized and unmineralized bone formation within the bone defect (Figure 5-16, Figure 5-17, Figure 5-18). In addition, the 1:1 MSC:EPC-seeded group displayed the highest and most significant levels of vascularization, with
the MSC- and EPC-seeded groups also displaying increased vascularization compared to the acellular group (Figure 5-19).

Figure 5-16. Representative histological images of Goldner’s Trichrome–stained longitudinal sections imaged under low, 4X, 10X, 20X and 40X magnification. Images shown for 4X magnification were taken from the region of interest highlighted in the low magnification image. Images shown for 10X, 20X, and 40X magnification are representative images of each group. Scale bar for low magnification images = 5 mm; Scale bar for 4X magnification images = 500 μm; Scale bar for 10X images = 500 μm; Scale bar for 20X images = 250 μm; 40X images = 100 μm.
Figure 5-17. Labeled Goldner’s Trichrome stained sections imaged under (A) 20X (scale bar = 250 µm), and (B) 40X (scale bar = 100 µm). M = microsphere scaffold,
C = unmineralized collagen, BV = blood vessel, O = osteoblast, Os = osteoid, B = mineralized bone.

Figure 5-18. Quantification of collagen formation within the rabbit ulnar defect. Ten regions on Trichrome stained sections were analyzed for the intensity of collagen (green color) from 3 samples in each group under 20X magnification with a color (RBG) histogram analysis tool from ImageJ software (arbitrary scale of 0 to 255, with 255 representing the highest green intensity).
Figure 5-19. Quantification of vessels within the rabbit ulnar defect. Vessels were counted in five regions on Trichrome stained sections under 10X magnification for 3 samples in each group.

5.4. Discussion

Insufficient vascularization of bone tissue engineered constructs hinders optimal bone regeneration. Here, we have demonstrated a two-pronged approach for enhanced vascularization, specifically involving the development of oxygen tension controlled constructs that allow for enhanced cell viability, as well as the optimal ratio of clinically-relevant cells for in vitro pre-vascularization, and enhanced ectopic bone and vascular formation in vivo.

In this study, we have utilized a previously described fabrication of porogen leaching and microsphere sintering to develop poly(lactic-co-glycolic acid) (PLGA)
microsphere scaffolds that demonstrate tunable oxygen tension and pH levels in the scaffold’s interior regions after long term *in vitro* cell culture. Specifically, by adding NaCl crystals to a mixture of PLGA microspheres, and leaching out the NaCl via soaking in water after thermal sintering the molded mixture, we developed biodegradable scaffolds with increased pore sizes and accessible pore volume, while retaining mechanical strength in the range of human cancellous bone.

With our newly-designed oxygen tension controlled matrices, we set forward to investigate the most effective cell ratio of MSC-derived osteoblasts and EPCs for *in vitro* pre-vascularization, and subsequent neo-vascularization and bone formation *in vivo*. We investigated the following MSC-derived osteoblasts/EPC ratios: 1 part MSC-derived osteoblasts to 4 parts EPC (1:4), 1 part MSC-derived osteoblasts to 1 part EPC (1:1), and 4 parts MSC-derived osteoblasts to 1 part EPC (4:1). We confirmed the maintenance of endothelial cell markers (i.e., CD31 and von Willabrand Factor (vWF)) by EPCs, and osteogenic cell markers (i.e., collagen type 1 and RunX2) by MSCs, cultured alone and in co-culture via immunofluorescence. After 2 days *in vitro*, the constructs of the various cells ratios, as well as the cell types alone, were implanted subcutaneously in SCID mice. Constructs seeded with MSC-derived osteoblasts and EPCs at a ratio of 1:1 demonstrated not only the highest level of vascularization throughout the implant construct, but also collagen formation after 8 weeks *in vivo*. Though constructs seeded with EPCs alone, as well as in co-culture with MSC-derived osteoblasts (ratio of 1:4) also demonstrated significant vascular formation throughout the construct compared to acellular constructs, it was significantly less than that of constructs seeded with the cells at a 1:1 ratio. In addition, significant collagen formation was observed throughout all the constructs that
were seeded with both cells types at all ratios (1:4, 1:1, 4:1), as well as constructs seeded with MSC-derived osteoblasts. However, again, constructs seeded with MSC-derived osteoblasts and EPCs at a ratio of 1:1 resulted in the highest levels of collagen formation. Vascular and bone formation in vivo was confirmed through immunostaining of endothelial markers CD31 and vWF, as well as osteogenic markers collagen type 1 and RunX2, respectively. Implanted acellular constructs demonstrated significant adipose tissue formation, as well as constructs seeded with only one cell type (i.e., EPCs-alone, MSC-derived osteoblasts-alone), as well co-culture ratio 1:4.

With this, we selected the ratio of 1:1 MSC:EPC as the most effective progenitor cell ratio, and pursued a load-bearing bone defect model. We evaluated the performance of 4 different groups, acellular scaffold constructs (i.e., negative control), MSC-seeded scaffold constructs, EPC-seeded scaffold constructs, and a co-culture of MSCs and EPCs at a ratio of 1:1. The co-culture group outperformed, and displayed the most significant increase in bone formation and vascularization with the bone defect as confirmed by MicroCT, and histological analysis. Furthermore, this group displayed the least amount of adipose and fibrous tissue formation, as compared to the acellular group, and MSC- and EPC- seeded group.

5.5. Conclusions

The fundamental implications of this study are apparent, and will lay the groundwork for future bone tissue engineering studies. We have developed oxygen tension-controlled matrices allowing for enhanced *in vitro* cell viability, as well the identified of the most effective cell ratio of MSC-derived osteoblasts to EPCs, two easily
accessible autologous cell sources. Though many studies have investigated co-implantation of MSC-derived osteoblasts and EPCs for effective neo-vascularization and bone formation, we were the first to examine the proper ratio of these two cell types. Further, we utilized both, optimally-porous oxygen tension-controlled matrices and the optimal ratio of effective progenitor cells for the enhanced repair and regeneration of a critically-sized load-bearing bone defect in rabbits.
6. CONCLUSIONS AND FUTURE DIRECTIONS

Large area or critically-sized bone defects represents a major clinical problem in orthopaedic and cranio-/maxillo- facial surgery (127). Large area bone defects may result from trauma, tumor resection, revision surgery and developmental deformities, and are unable to heal spontaneously (4). Repair of large defects require bone grafts/graft substitutes that can physically support bone regeneration, while providing surface area for cell attachment and tissue growth. Current treatment options for large area bone defects include bone grafts, distraction osteogenesis, demineralized bone matrix, and porous hydroxyapatite; all of which have been associated with significant challenges and complications (5-10). Thus, there is a warranted search for better bone replacement methods to overcome the drawbacks of the currently used bone graft materials.

Bone tissue engineering research has revealed tremendous potential for the treatment of bone defects. The bone tissue engineering paradigm classically involves the combination of one or more of the following components: a mechanically-compatible scaffold, effective cell populations and/or growth factors. However, central necrosis or lack of bone tissue formation due to the lack of or insufficient vascularization of bone constructs is a well-recognized obstacle to the success of complete bone regeneration and host integration in bone tissue engineering (35).

In the present study, we have demonstrated a two-pronged approach involving the development of optimally-porous constructs that allow for enhanced cell viability, as well as the identification of effective clinically-relevant cells for enhanced bone formation and vascularization. Again, in the first component of our approach, we developed a novel optimally-porous biodegradable scaffold for bone regeneration and vascularization. We
fabricated poly(D,L-85 lactide-co-15 glycolide) (PLGA) microsphere scaffolds with increased porosity via the combination of thermal sintering and porogen (i.e., NaCl particles) leaching. Specifically, we combined PLGA microspheres with NaCl, thermally sintered, and then leached out the NaCl by soaking the constructs in water. Through this method, scaffold porosity and mechanical properties may be tuned according to the clinical requirement by controlling the size and amount of the porogen added during the fabrication process.

Through this method, we have improved PLGA microsphere scaffold performance and its ability to support osteoblast cell survival, proliferation and mineralization throughout the construct, and yet retained mechanical compatibility for effective bone regeneration. We determined PLGA microsphere scaffolds fabricated with 20% NaCl to be *optimally-porous*. Although optimally-porous scaffolds displayed decreased mechanical strength as compared to the control scaffolds (i.e., fabricated without porogen), the mechanical strength of these scaffolds remained within the mechanical constraints of human cancellous bone. However, unlike control scaffolds with limited pore sizes and accessible pore volume, optimally-porous scaffolds displayed significantly more pores sizes in the range of 200-400 µm, which has been previously cited as a critical pore size range for vascularization in bone tissue engineering constructs. Optimally-porous scaffolds also displayed increased oxygen levels and near-normal pH levels. These scaffolds proved to be fully osteoconductive as they supported pre-osteoblast cell infiltration, proliferation, and survival throughout the entire scaffold *in vitro*. Furthermore, optimally-porous PLGA microsphere scaffolds displayed enhanced and homogenous mineralization *in vitro*. 
In addition, we demonstrated the ability to modulate scaffold porosity and in turn, develop oxygen tension controlled matrices that may be effective for large area bone regeneration. Specifically, increasing the concentration of porogen during scaffold fabrication resulted in a systematic increase in not only porosity, but also available oxygen tension throughout the matrix. The enhanced survival, proliferation, differentiation and mineralization of pre-osteoblasts may be attributed to the increase in available oxygen tension. Thus, the proposed optimally-porous scaffolds with improved oxygen availability and bone compatible mechanical properties are desirable for large area bone regeneration. Oxygen tension control via scaffold porosity optimization may provide opportunities in designing next generation scaffold systems most effective for large area/ critical sized bone defect repair.

As for the second component of our two-pronged approach, we aimed to identify effective clinically relevant cell populations for enhanced bone formation and vascularization. We examined endothelial progenitor cells (EPCs) as an effective endothelial population since they have been previously proven to promote vascularization and in turn, promote successful bone formation at the graft site. Additionally, EPCs display high angiogenic, proliferative and survival potential in situ.

We have, for the first time, sought to identify the ideal isolation source for EPCs, either bone marrow or peripheral blood (BM-EPCs or PB-EPCs), that would yield the most effective EPC cell population for enhanced vascularization and bone formation. PB-EPCs outperformed BM-EPCs on all measures. PB-EPCs displayed typical endothelial cell markers, such as CD31, as well as high angiogenic potential in three-dimensional extracellular matrix in vitro. Furthermore, PB-EPCs cultured
simultaneously with mesenchymal stem cells (MSCs), displayed significantly enhanced expression levels of key osteogenic and vascular markers, including alkaline phosphatase, bone morphogenetic protein 2, and vascular endothelial growth factor. On the contrary, BM-EPCs did not express CD31, and instead, expressed key smooth muscle markers. BM-EPCs further failed to display vasculogenic activity. Therefore, we identified the highly angiogenic peripheral blood derived-EPCs (PB-EPC) as an ideal autologous cell population since their isolation does not risk donor site morbidity, and importantly, they effectively result in enhanced vascularization and success of engineered bone tissue regeneration.

With the fabrication of newly-designed optimally-porous scaffolds and identification of effective progenitor cells, we set forward to investigate the most effective cell ratio of MSC-derived osteoblasts and EPCs for in vitro pre-vascularization, and subsequent neo-vascularization and bone formation in vivo. We determined that constructs seeded with MSC-derived osteoblasts and EPCs at a ratio of 1:1 demonstrated not only the highest level of vascularization throughout the implant construct, but also collagen formation 8 weeks post-implantation in a mouse subcutaneous model. Vascular and bone formation in vivo was confirmed through immunostaining of endothelial markers CD31 and vWF, as well as osteogenic markers collagen type 1 and RunX2, respectively. With this, we selected the ratio of 1:1 MSC:EPC as the most effective progenitor cell ratio, and pursued a load-bearing, critical-sized bone defect model. We evaluated the performance of 4 different groups, acellular scaffold constructs (i.e., negative control), MSC-seeded scaffold constructs, EPC-seeded scaffold constructs, and a co-culture of MSCs and EPCs at a ratio of 1:1. The co-culture group outperformed, and
displayed the most significant increase in bone formation and vascularization with the bone defect as confirmed by MicroCT, and histological analysis. Furthermore, this group displayed the least amount of adipose and fibrous tissue formation, as compared to the acellular group, and MSC- and EPC- seeded group.

As a whole, this investigation presents a landmark study, and may lay the groundwork for future bone tissue engineering studies to make steps even closer to achieving clinical success in bone regeneration and repair. We have developed optimally-porous and oxygen tension-controlled scaffolds allowing for enhanced in vitro cell viability, as well the identified of the most effective cell ratio of MSC-derived osteoblasts to EPCs, two easily accessible autologous cell sources. Further, we utilized both, optimally-porous oxygen tension-controlled matrices and the optimal ratio of effective progenitor cells for the enhanced repair and regeneration of a critically-sized load-bearing bone defect in rabbits. Future studies should focus on adopting the fundamental concepts presented in this investigation (i.e., scaffold and autologous cell population) into a even more clinically-tailored concept. For example, more effective cell isolation, seeding and culturing methods need to be used in order to streamline the engineering process, and decrease the safety risks associated with the handling the constructs during the pre-implantation period. Bioreactors that can combine all three steps may be used for this purpose, and may drive the way for safer and more effective bone tissue engineering. In addition, the incorporation of immunomodulatory strategies may be used to modulate the host’s foreign-body response (i.e., fibrous tissue encapsulation), an event that is often observed to be an inhibitory factor for optimal tissue regeneration and integration.

In conclusion, bone tissue engineering research has recently gained significant
momentum and revealed tremendous potential for the treatment of bone defects, and prospects for achieving clinically successful bone regeneration are extremely optimistic.
7. APPENDIX 1 – Protocols

7.1. Fabrication of PLGA Microspheres

7.1.1. Materials

- Poly(D,L-85 Lactide-co-15 glycolide) (PLGA) (Lakeshore Biomaterials; Birmingham, AL)
- Poly (vinyl alcohol) (PVA) (Sigma)
- Methylene chloride (CH₂Cl₂) (Sigma)

7.1.2. Protocol

85:15 PLGA polymer was dissolved in organic solvent, Methylene Chloride at 4:1 ratio and vortexed until it dissolved. The mixture was then slowly added to a 1% poly (vinyl alcohol) solution while stirring at 250 rpm and left stirring over night at 300 rpm. The microspheres were then vacuum dried for 24 hours then sieved to obtain 425-600 µm size range and kept in desiccator until needed.

7.2. Fabrication of Optimally-Porous PLGA Microsphere Scaffold

7.2.1. Materials

- PLGA microspheres (425-600 µm diameter)
- Porogen, NaCl crystals (200-300 µm diameter)
• Steel mold to fabricate disc-shaped (10 mm diameter, 2 mm height) or cylinder-shaped (5 mm diameter, 15 mm or 10 mm height) scaffolds
• Oven set to 100°C

7.2.2. Protocol

Control scaffolds were fabricated by packing approximately 0.15 g of PLGA microspheres (425-600 µm diameter) into each compartment within the steel mold. Scaffolds with increased porosity were fabricated by mixing PLGA microspheres (425-600 µm diameter) and NaCl crystals (200-300 µm diameter) at specific weight ratios (i.e., PLGA:NaCl ratios include 90:10, 80:20, 70:30, 60:40, 50:50), which was then packed in the the steel mold in a similar manner as described with the control scaffolds. The mold was then subjected to heat treatment of 100°C for 1 hour to form three-dimensional scaffolds. The mold was then allowed to cool to room temperature before the scaffolds were removed from the mold. Scaffolds were then soaked in water for 2 hours in order to leach out the NaCl. Scaffolds were then stored in a desiccator until further use.

7.3. Mechanical Testing

7.3.1. Materials

• Pre-fabricated PLGA microsphere scaffolds (10 mm height x 5 mm diameter)
• Instron model 5544
• Computer with installed Merlin data analysis software

7.3.2. Protocol
Cylindrical scaffolds (n = 6) with 2:1 aspect ratio (10 mm length and 5 mm diameter) were used for mechanical characterization. Compressive testing will carry out using an Instron model 5544 with a cross head speed of 2 mm/min maintained until the sample failed. We followed the standard protocol of ASTM 1621.37. The compressive modulus and maximum compressive strength of scaffolds were determined using the Merlin data analysis software. Compressive strength was defined as the maximum stress magnitude. Apparent modulus was measured as the tangential slope of the linear region of the effective stress–strain curve at 50% of compressive strength magnitude.

7.4. MicroCT Analysis

7.4.1. Materials
• Pre-fabricated PLGA microsphere scaffolds (2 mm height x 10 mm diameter)
• Cone-beam micro-focus X-ray computed tomography (mCT40; Scanco Medical AG)

a. Protocol
Scaffold specimens were imaged using cone-beam micro-focus X-ray computed tomography (CT) to render three-dimensional (3D) models for
direct quantitation of porosity (mCT40; Scanco Medical AG). Serial
tomographic images were acquired at 45kV and 177mA, collecting 2000
projections per rotation at 300 millisecond integration time. Three-
dimensional 16-bit grayscale images were reconstructed using standard
convolution back-projection algorithms with Shepp and Logan filtering, and
rendered within a 12.3-mm field of view at a discrete density of 4,629,630
voxels/mm3 (isometric 6 mm voxels). Segmentation of solid scaffold from
open porosity was performed in conjunction with a constrained Gaussian
filter to reduce noise, applying a threshold of -220 Hounsfield units (water =
0, air = -1000). Direct measurements of internal porosity included volume
fraction, size, connectivity, accessible internal pore volume, and accessible
solid surface area of the scaffold (as a function of pore dimension). The
accessible volume and surface parameters provide direct measurements of the
pore volume and the surface available to cell infiltration as a function of
minimum pore dimension, using a distance transformation algorithm similar
to that used by Moore et al.

7.5. Scanning Electron Microscopy (SEM) Using the
FEI Strata 400s Dual Beam FIB

7.5.1. Materials

- PLGA microsphere scaffolds
- Gold/palladium
7.5.2. Protocol

The morphology of the PLGA microsphere scaffolds was analyzed using the FEI Strata 400s Dual Beam FIB. The surface characterization of both microspheres and scaffolds was done in the SEM mode (2KeV). Samples were prepared by coating with gold/palladium (1-2 minutes) and examined under SEM.

7.6. Scaffold Sterilization

7.6.1. Materials

- 70% ethanol
- PBS
- UV radiation

7.6.2. Protocol

Scaffolds were sterilized by immersing in 70% ethanol for 30 minutes. Scaffolds were then washed three times in sterile PBS before exposing them to UV radiation, 30 minutes each side of scaffold. Scaffolds were then placed in 24 well plates and let to dry for few minutes.

7.7. Cell Culture
7.7.1. Cell Types

7.7.1.1. MC3T3 Pre-Osteoblast Cells
Pre-osteoblastic MC3T3-E1 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). Cells from Passage 18-23 were used for experiments.

7.7.1.2. Rabbit Mesenchymal Stem Cells (MSCs)

7.7.1.2.1. Materials

- Betadine Solution
- 100% Ethanol
- Phosphate Buffered Saline (PBS) Solution
- Isolation Medium (a-MEM + 10% Fetal Bovine Serum + 1% Pen/Strep)
- Growth Medium (a-MEM + 15% Fetal Bovine Serum + 1% Pen/Strep)
- Lymphoprep (1.077 g/mL)
- Sterile Drapes and Sponges
- Scalpel
- Sterile Petri Dishes (100 x 15 mm)
- Sterile Disposable Syringes with Luer Lock Sterile Needles (16-, 18- and 21-G)
- Sterile glass beaker (100 mL)
7.7.1.2.2. Protocol

The isolated long bones of New Zealand white rabbits were placed in a beaker containing 100% EtOH for 5 minutes, and then in individual 50 mL centrifuge tubes containing isolation media. The bones were kept on ice until ready for marrow isolation. The long bones were removed from media and place into a 100 mm sterile Petri dish in a sterile cell culture hood. The bones were rinsed with PBS, and any adherent muscle or soft tissues were removed from the long bones using scalpel and scissors. The long bones were rinsed again with PBS and transferred to a clean 100 mm sterile Petri dish. The metaphyses (flared ends of long bone) of each bone were removed using a bone cutter to expose the marrow cavity. The long bone pieces were placed into individual sterile 50 mL polystyrene test tubes, and 5 milliliters of isolation medium (containing 1250 U heparin) was used to flush the marrow contents repeatedly using a 5 mL syringe containing a 21gauge needle. The marrow contents were centrifuged at 1000 x g (2400 rpm) for 5-10 minutes at 4°C. The supernatant (containing mainly bone marrow fat and isolation medium) was removed, and the cell pellet was re-suspended in 5 mL of isolation medium, and then 5 ml of Lymphoprep was added to a new 15 milliliter sterile centrifuge tube, and the cell suspension was layered onto the Lymphoprep layer and
centrifuged at 400 x g (1500 rpm) for 30-40 min at 18-20°C. Centrifugation resulted in 4 different layers: (i) the bottom is predominately red blood cells/granulocytes, (ii) above is a clear Ficoll layer, (iii) above this is a slightly pink hazy layer containing the majority of the mononuclear cells, and (iv) the uppermost is predominantly plasma, platelets and PBS. The uppermost layer was aspirated taking care to avoid disturbing the mononuclear layer. The mononuclear layer and two-thirds of the Ficoll layer was transferred to new 50-ml tubes, and centrifuged at 600-750 rpm for 10 minutes at 18-20°C. The supernatant was aspirated and the pellet was washed twice with PBS, and then centrifuged at 500 g for 5 minutes. The cells were resuspended in growth medium, and then plated at an appropriate seeding density on TCPS culture flasks, and cultured at 37°C in 5% CO₂. Non-adherent cells were removed after 24 hours by washing with PBS. The basal media was changed subsequently every 4 days, until culture reached 90% confluency (2 weeks). MSCs were recovered using 0.25% Trypsin-EDTA and re-plated at a density of 5,000-6,000 cells per cm² of surface area as passage 1 (P1) cells.

7.7.1.3. Rabbit Bone Marrow-Derived Endothelial Progenitor Cells (BM-EPCs)
7.7.1.3.1. Materials

- Betadine Solution
- 100% Ethanol
- Phosphate Buffered Saline (PBS) Solution
- Sterile Drapes and Sponges
- Scalpel
- Sterile Petri Dishes (100 x 15 mm)
- Sterile Disposable Syringes with Luer Lock
- Sterile Needles (16-, 18- and 21-G)
- Sterile glass beaker (100 mL)
- Lymphoprep (1.077 g/mL)
- Endothelial Growth Media-2 (EGM-2, Lonza)
- 150 mm plates coated with Rabbit Type 1 Collagen (1 µg/cm²)
- 1 plate / 50 ml of collected blood
- 0.25% Trypsin/EDTA

7.7.1.3.2. Protocol

Bone marrow from the long bones of New Zealand White Rabbits (4-5 kg) were be isolated, mixed with an equal volume of PBS and centrifuged on a Lymphoprep (1.077 g/ml) gradient for 400 x g (1500 rpm) for 30-40 min at 18-20°C. The mononuclear fraction was isolated, and then washed three times with PBS. The mononuclear fraction was cultured on
150 mm culture dishes coated with 1 µg/cm² of rabbit type I collagen in EGM-2 Endothelial Medium (EGM-2) at 37°C and 5% CO2. After 4 days of culture, non-adherent cells were removed by washing with PBS, and a fresh medium was applied. When cells reached about 90% confluency, cells were trypsinized, and passaged. The cells at passage 2-4 were used for cell characterization and construct engineered bone.

7.7.1.4. **Rabbit Peripheral Blood-Derived Endothelial Progenitor Cells (PB-EPCs)**

7.7.1.4.1. **Materials**

- Anesthesia (ketamine (90-120 mg/kg body wt) and xylazine (5-10 mg/kg body wt) given via intramuscular injection)

- BD Vacutainer Chemistry Tubes - 10 mL Plasma Tubes with Spray-Coated Sodium Heparin (BD, #02-689-6)

- BD Vacutainer Tube Holder (BD, #22-289-953)

- BD Vacutainer Blood Collection Needles 21 x 1-1/2 in. (BD, #266521)

- 70% ethanol

- Lymphoprep

- Phosphate Buffered Saline (PBS) Solution (1x)

- Endothelial Growth Media-2 (EGM-2, Lonza)
• 150 mm plates coated with Rabbit Type 1 Collagen (1 µg/cm²)
• 1 plate / 50 ml of collected blood
• 0.25% Trypsin/EDTA

7.7.1.4.2. Protocol
Peripheral blood was obtained via terminal exsanguination of a New Zealand White rabbit (4-5 kg). The rabbits were anesthetized with intramuscular ketamine (90-120 mg/kg body wt) and xylazine (5-10 mg/kg body wt). The rabbit was positioned in dorsal recumbency, and chest area was wiped with 70% ethanol. An incision overlying the thoracic cavity was made to expose the heart. Blood collection was performed by puncturing the heart with a 21 guage needle connected to a Vacutainer blood collection system. Once blood withdrawal was complete, sodium pentobarbital is injected intracardiac at 100 mg/kg to ensure death.

Rabbit peripheral blood mononuclear cells (PBMNCs) was isolated from ~200 ml of peripheral blood by density gradient centrifugation with Lymphoprep separation solution (d=1.077 g/ml). Specifically, 15 milliliters of Lymphoprep was added to each 50-ml centrifuge tube. Then, an equal amount of PBS was added to the peripheral blood, and 35 milliliters of blood/PBS solution was slowly pipetted into each 50-ml
centrifuged at 400 g or 20 min at room temperature. This resulted in four visible layers after centrifugation: (i) the bottom is predominately red blood cells, (ii) above is a clear Lymphoprep layer, (iii) above this is a slightly pink and hazy layer containing the majority of the mononuclear cells, and (iv) the uppermost is predominately PBS. The uppermost layer was aspirated taking care to avoid disturbing the mononuclear layer. The mononuclear layer and two-thirds of the Ficoll layer was transferred to new 50-ml tubes, and PBS was added to fill each tube and spin at 1500 g for 5 min to pellet cells. The pellet was washed once with PBS, resuspend in EGM-2 media, and then plated mononuclear cells from each 50 ml collected blood onto one 100 mm collagen-coated plate. The cells were cultured at 37°C and 5% CO₂ for 4 days, and then non-adherent cells removed by washing with PBS two times. The culture was maintained for another 4–5 weeks, and media was be changed every 3 days. After reaching about 80% confluence, cells were trypsinized and seeded into 150 mm culture plate coated with rabbit Type I collagen for expansion. EPCs from passage 2–4 were used for experiments.
7.7.1.5. Rabbit Vascular Smooth Muscle Cells (SMCs)

7.7.1.5.1. Materials
- Betadine Solution
- Isopropyl Alcohol
- 100% Ethanol
- Phosphate Buffered Saline (PBS) Solution
- Sterile Drapes and Sponges
- Scalpel, forceps
- Sterile Petri Dishes (100 x 15 mm)
- Sterile glass beaker (100 mL)
- DMEM + 3X Penicillin/Streptomycin
- 0.25% Trypsin/EDTA
- 7 milliliters of digesting solution (10mL of DMEM+PenStrep containing 15 U/mL Elastase (Worthington), 200 U/mL collagenase Type 2 (Worthington), and 1.7 mg/mL of bovine serum albumin (BSA))
- 0.22-um low-protein retention filter (Millipore, Billerica, MA))

7.7.1.5.2. Protocol
In a New Zealand white rabbit, the thoracic aorta was isolated from its origin just above the heart to the iliac bifurcation, and then transferred to a 50 ml test tube containing DMEM + 3x
penicillin/ streptomycin 37 °C. The vessels were rinsed three times with DMEM + 3X Pen/Strep at 37°C, and then the connective tissues and adventitia was gently removed from the aorta using forceps. The aorta was washed in DMEM with 3X Pen/Strep at 37°C by repeatedly pipetting out with a wide-mouthed, glass pipet to ensure the removal of blood within the aorta. The aorta was chopped into small pieces, and then was added to 7mL of digesting solution (10mL of DMEM+PenStrep containing 15 U/mL Elastase (Worthington), 200 U/mL collagenase Type 2 (Worthington), and 1.7 mg/mL of bovine serum albumin (BSA). This solution was filtered through a 0.22-um low-protein retention filter (Millipore, Billerica, MA)) and incubated at 37 °C for about 60–75 min with gentle shaking. The completion of digestion is indicated by homogenization of the aorta in the digestion media. The solution was then spun at 1000 g for 5 min, and then the supernatant was gently discarded. The pellet was washed by swirling gently (slight dissociation) with 5 milliliter glass pipette three times with DMEM/PenStrep (5 milliliters). The supernatant containing single cells was spun at 100 g for 30 seconds, and then the cells were plated on culture dishes containing cell culture media, and culture at 5% CO₂ and 95% air.
7.7.1.6. **Human Umbilical Vein Endothelial Cells (HUVECs)**
HUVECs were obtained from American Type Culture Collection (#RL-2873, ATCC, Manassas, VA). Cells from Passage 4-6 were used for experiments.

7.7.2. **Cell Culture Mediums**

7.7.2.1. **Basal Growth Medium**
Basal growth medium for MC3T3 cells, MSCs and SMCs is DMEM supplemented with 10% FBS and 1% penicillin/streptomycin.

7.7.2.2. **Osteogenic Medium**

7.7.2.2.1. MC3T3 Cells
Cells were cultured in DMEM supplemented with 10% FBS, 1% penicillin-streptomycin, 3mM β-glycerophosphate and 10 µg/ml ascorbic acid.

7.7.2.2.2. Mesenchymal Stem Cells
For osteogenic differentiation, the culture medium (DMEM, 10% FBS, 1% Penicillin/Streptomycin) was supplemented with 10⁻⁸ M dexamethasone, 10mM β-glycerophosphate, and 50 µg/ml ascorbic acid.
7.7.2.3.  **Endothelial Growth Medium**
BM-EPCs, PB-EPCs and HUVECs were cultured in EGM-2 media (CC-3162, Lonza). EGM-2 includes endothelial basal medium (EBM2) supplemented with 10% FBS, 1% Penicillin/Streptomycin, and EGM2 Singlequots (hEGF,VEGF, hFGF-B, R3-IGF-1, ascorbic acid, heparin, gentamicin–amphotericin-B.

7.7.2.4.  **1-to-1 Mix of Osteogenic And Endothelial Medium**
7.7.2.4.1.  Co-Culture of Mesenchymal Stem Cells and Endothelial Progenitor Cells
Co-cultures of MSCs and EPCs were cultured in a 1-to-1 mix of osteogenic and endothelial medium (i.e., 1 part osteogenic media (DMEM, 10% FBS, 1% Penicillin/Streptomycin, 10^{-8} M dexamethasone, 10 mM β-glycerophosphate, and 50 ug/ml ascorbic acid) and 1 part endothelial media (EGM-2 media, 10% FBS, 1% Penicillin/Streptomycin, EGM2 Singlequots)).

7.7.3.  **Cryopreservation Of Cells**

7.7.3.1.  **Materials**
- Dimethyl sulfoxide (DMSO, Sigma-Aldrich, D4540)
- Cryogenic vials
- 0.25% Trypsin-EDTA (Sigma-Aldrich, T4049)
- 15ml centrifuge tube
• 70% isopropyl alcohol (inside the cryogenic storage container)
• PBS Phosphate-Buffered Saline (Gibco)
• Cryogenic storage container

7.7.3.2. **Protocol**
To cryopreserve cells for later use, the cells were trypsinized and transferred to a 15 milliliter centrifuge tube, and centrifuged at 500 rpm for 10 minutes. The cells were resuspended in 5 milliliters of media, and counted using trypan blue and a hemacytometer for a viable cell count. In each cryopreservation 1.5 milliliter tube, 0.4 ml FBS, 0.1 ml DMSO, and 1x10^6 cells/0.5 ml media was added to create a cell suspension of 1x10^6 cells per milliliter. The cells were then immediately transferred to cryogenic storage container at -80°C overnight, before permanent storage in liquid nitrogen.

7.8. **Cell Seeding on Scaffolds and Culture Conditions**

After cell trypsinization, a cell suspension was uniformly seeded onto the scaffolds. The disc-shaped scaffolds were placed flat on the culture plate, and a 20 μL cell suspension was uniformly added to the top of the scaffold. The cylindrical scaffolds were placed along the length of the scaffold on the culture plate, and a 40 μL cell suspension was added to the lengthwise surface as the scaffold was slowly rotated (i.e., along the long axis of the scaffold) to maintain uniform cell seeding. The
cell-seeded scaffolds were incubated for 2 hour at 37°C to allow for cell adhesion onto the scaffolds before 1 milliliter of media was added. Cell culture was maintained for pre-determined time-points in an incubator at 37°C, 5% CO₂, and 95% humidified air.

7.9.  **Cell Seeding Efficiency**

7.9.1.  **Materials**

- Cell suspension
- Hemocytometer
- Trypan blue
- Light microscope

7.9.2.  **Protocol**

After 6 hours of cell seeding, scaffolds were transferred to new wells. Cells at the bottom of the original wells were trypsinized, resuspended, and counted with a hemocytometer. The cell-seeding efficiency (i.e., the number of cells that adhered to the scaffolds) was determined by the difference between the number of cells initially seeded and the number of cells that were counted at the bottom of the well.

7.10.  **Cell Proliferation (PicoGreen Analysis)**

7.10.1.  **Materials**

- DNA solution in 1% Triton X100 solution
• Quant-iTTM PicoGreen® dsDNA Assay Kit (Molecular Probes, #P7589)
• PicoGreen dsDNA quantitation reagent (Component A)
• Buffer, 20X TE (Component B)
• Lambda DNA standard (Component C)
• Sterile, distilled, DNase-free water

7.10.2. Protocol
To quantitate dsDNA (double stranded DNA), we utilized the Quant-iTTM PicoGreen® dsDNA Assay Kit. PicoGreen dsDNA quantitation reagent is an ultrasensitive fluorescent nucleic acid stain for quantitating dsDNA in solution. To prepare sample, the experimental DNA solution was diluted in TE to a final volume of 1.0 milliter in test tubes. 1.0 mL of the aqueous working solution of the Quant-iTTM PicoGreen® reagent was added to each sample, and then incubated for 2-5 minutes at room temperature in the dark. The standards were prepared as indicated below. The fluorescence of the unknown samples and standards were measured using the TECAN at a wavelength of 523 nm. The fluorescence value of the reagent blank was subtracted from that of each of the samples. The DNA concentration of the sample was determined from the standard curve generated in DNA Standard Curve.

High Range Standard curve for λDNA:
<table>
<thead>
<tr>
<th>Volume (µL) of 2µg/µL λDNA</th>
<th>Volume (µL) of 1X TE (diluted B)</th>
<th>Volume (µL) of diluted A</th>
<th>Final concentration of λDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1000</td>
<td>1000</td>
<td>0ng/µL (blank)</td>
</tr>
<tr>
<td>1</td>
<td>999</td>
<td>1000</td>
<td>1 ng/µL</td>
</tr>
<tr>
<td>10</td>
<td>990</td>
<td>1000</td>
<td>10 ng/µL</td>
</tr>
<tr>
<td>25</td>
<td>975</td>
<td>1000</td>
<td>25 ng/µL</td>
</tr>
<tr>
<td>50</td>
<td>950</td>
<td>1000</td>
<td>50 ng/µL</td>
</tr>
<tr>
<td>100</td>
<td>900</td>
<td>1000</td>
<td>100 ng/µL</td>
</tr>
<tr>
<td>250</td>
<td>750</td>
<td>1000</td>
<td>250 ng/µL</td>
</tr>
<tr>
<td>500</td>
<td>500</td>
<td>1000</td>
<td>500 ng/µL</td>
</tr>
<tr>
<td>1000</td>
<td>0</td>
<td>1000</td>
<td>1000 ng/µL</td>
</tr>
</tbody>
</table>

### 7.11. Alkaline Phosphatase Assay

#### 7.11.1. Materials

- Sample in 1% Triton X100
- BioRad Alkaline Phosphatase Substrate Kit (Bio-Rad Laboratories, Inc)
- Diethanolamine Buffer (5X) (make 1X soln from 5X soln: 1ml 5X soln: 4ml H2O=1X)
- Note: to make 0.4 N sodium hydroxide solution (dissolve 2.0g sodium hydroxide in 125 ml of DDI water)
b. **Protocol**

Alkaline phosphatase activity of cells cultured on microsphere scaffolds were measured as an early marker for osteogenic phenotype using an alkaline phosphatase (ALP) substrate kit (Bio-Rad, Hercules, CA). In this assay, the early phenotypic marker ALP, from osteoblasts in culture, converts p-nitrophenyl phosphate (p-NPP) into p-nitrophenol (p-NP). The rate of p-NP formation is directly proportional to the ALP activity and can be measured colorimetrically. At pre-determined timepoints, the cell-scaffold constructs were washed twice with PBS to remove any unattached cells. These scaffolds were then frozen in a −70°C with 1 mL of 1% Triton X100. At the end of the cell study, samples from all time points were subjected to three freeze–thaw cycles and collected the cell lysate for ALP assay. For a volume of 100 µL of cell lysate, a 400 µL of p-NPP substrate solution and buffer mixture was added and incubated at 37 °C for 30 min. The reaction was stopped by adding 500 µL of 0.4 N NaOH. Subsequently, the ALP induced p-NP production can be estimated by measuring the absorption at 405 nm the TECAN. The results of ALP activity will be normalized by the total protein or DNA (i.e., BCA assay or DNA Picogreen Assay) from each individual sample.
7.12. Bicinchoninic Acid (BCA) Total Protein Assay

7.12.1. Materials

- BCA Protein Assay Reagent (Bicinchoninic Acid) (Peirce, #23225)
  - Kit Contents: BCA Reagent A, 2 x 500 mL; BCA Reagent B, 25 mL; Albumin Standard Ampules, 2mg/mL, 10 x 1mL
- 1% Triton X100 (Sigma-Aldrich, # X100-100ML)

7.12.2. Protocols

In order to normalize specific protein levels of cells cultured on scaffolds, we determined total protein levels via the Bicinchoninic Acid Assay (BCA) total protein assay. At each time point, the media was aspirated off, and the scaffolds were washed once with PBS. The cells were lysed with 1% Triton X100 (1 milliliter/sample), and two freeze-thaw cycles. The working reagent was prepared by mixing 50 parts of BCA reagent A with 1 part of BCA reagent B. In a 48 well plate, 100 µl of each unknown sample or standard (i.e., diluted with 1% Triton X100 solution) was pipetted, and then 800 µl of the working reagent was added to each well, and mixed thoroughly. The plate was covered and incubated at 37°C for 30 minutes, and then the absorbance was measured at 550 nm on Tecan.

<table>
<thead>
<tr>
<th>Vial</th>
<th>Volume of</th>
<th>Volume &amp; Source</th>
<th>Final BSA</th>
</tr>
</thead>
</table>

Diluted albumin (BSA) standards:
<table>
<thead>
<tr>
<th>diluent (μl)</th>
<th>of BSA</th>
<th>concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>450</td>
<td>150 μl of stock</td>
</tr>
<tr>
<td>B</td>
<td>300</td>
<td>300 μl of A</td>
</tr>
<tr>
<td>C</td>
<td>300</td>
<td>300 μl of B</td>
</tr>
<tr>
<td>D</td>
<td>360</td>
<td>240 μl of C</td>
</tr>
<tr>
<td>E</td>
<td>300</td>
<td>300 μl of D</td>
</tr>
<tr>
<td>F</td>
<td>320</td>
<td>80 μl of E</td>
</tr>
<tr>
<td>G</td>
<td>400</td>
<td>0</td>
</tr>
</tbody>
</table>

7.13. Alizarin Red Mineralization Assay

7.13.1. Materials

- 4 M Alizarin Red, pH 4.23: add 1.369g of powder dye to 100 ml of DDI water, use 1N NaOH to adjust pH.
- 10% (w/v) Cetylpyridinium Chloride (CPC), pH 7.0: add 10g to 100ml of 10mM Sodium Phosphate Na₂PO₄. Use 1N HCl to adjust pH
- 10 mM Na₂PO₄: dissolve 0.142 in 100 mL
- 70% Ethanol
- PBS w/o Ca or Mg
7.13.2. Protocol

Mineralized matrix synthesis was evaluated using Alizarin Red staining method for calcium deposition. This colorimetric analysis is based on solubility of the red matrix precipitate with cetylpyridinium chloride (CPC) to yield a purple solution. At predetermined time points, cell-scaffold constructs were rinsed free of media with DDI water and fixed for 1 hour with 70% ethanol at 4°C. Ethanol was removed and constructs were air-dried for 5–10 minutes. The samples were washed one time with DDI water, and then covered with 500 μL alizarin red dye for 10 minutes at room temperature. Samples were then washed with DDI water 5 or more times until no color can be washed out. Next, 1 milliliter of 10% CPC was added on cellularized scaffolds and incubated at room temperature for 30 minutes at which point the color will be stable. The absorbance was read on a plate reader at 562 nm using TECAN. Samples can be diluted 1:10 in additional CPC if necessary (if the machine reads “OVER” you can dilute).

7.14. Histology


7.14.1.1. Materials

- Formalin-fixed samples
- Ethanol (70%, 95%, 100%)
- Histoclear (National Diagnostics, #HS-200)
- Paraplast (Fisher, #23-021-401)


After 24 hours of formalin fixation, samples were processed through the following steps for paraffin-embedding:

<table>
<thead>
<tr>
<th>Solution</th>
<th>Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>70% ethanol</td>
<td>20 min</td>
</tr>
<tr>
<td>95% ethanol</td>
<td>20 min</td>
</tr>
<tr>
<td>95% ethanol</td>
<td>20 min</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>20 min</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>20 min</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>20 min</td>
</tr>
<tr>
<td>Histoclear</td>
<td>30 min</td>
</tr>
<tr>
<td>Histoclear</td>
<td>30 min</td>
</tr>
<tr>
<td>Paraffin</td>
<td>30 min, 65°C</td>
</tr>
</tbody>
</table>

7.14.2. Poly(Methylmethacrylate) (PMMA) Slow Embedding & Sectioning

7.14.2.1. Materials
- Formalin-fixed samples
- Ethanol (70%, 95%, 100%)
- Toluene
- Methylmethacrylate (MMA)
- Dibutyl phthalate (DP) containing increasing concentrations of the catalyst benzoyl peroxide (BP)
- Tungsten carbide knife
- Chromalum-gel coated slides

7.14.2.2. **Protocol**

The formalin-fixed bones were stripped of soft tissue and placed directly in 70% ethanol at 4°C for at least 24 hours. (Bones can be stored for up to one month at 4°C in 70% ethanol). After 24 hours, bones were processed in a tissue processor and dehydrated and cleared according to the following schedule:

<table>
<thead>
<tr>
<th>Solution</th>
<th>Time(hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>70% ethanol</td>
<td>1.5</td>
</tr>
<tr>
<td>95% ethanol</td>
<td>1.5</td>
</tr>
<tr>
<td>95% ethanol</td>
<td>2.0</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>3.0</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>4.0</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>4.0</td>
</tr>
<tr>
<td>100% ethanol:toluene (50:50)</td>
<td>4.0</td>
</tr>
<tr>
<td>Toluene</td>
<td>3.5</td>
</tr>
<tr>
<td>Toluene</td>
<td>2.0</td>
</tr>
<tr>
<td>Toluene</td>
<td>2.0</td>
</tr>
</tbody>
</table>
Bones were subjected to alternating cycles of pressure and vacuum on the tissue processor in each solution above. Following dehydration and clearing, bones were infiltrated with a mixture of 85% methylmethacrylate (MMA) and 15% dibutyl phthalate (DP) containing increasing concentrations of the catalyst benzoyl peroxide (BP) as follows: MMA I (3 days): 85% MMA, 15%DP; MMA II (3 days): 85% MMA, 15% DP, 1% BP; MMA III (3 days): 85% MMA, 15% DP, 2.5% BP. Each MMA solution was stirred at least two hours prior to use. All infiltration was carried out at 4°C.

Bases for embedding were prepared in 20 ml scintillation vials containing 3 ml of MMA III polymerized in a 37°C radiant heat oven. Once infiltration was complete, bones were placed on a pre-polymerized base of MMA III, covered with freshly made MMA III, capped tightly and allowed to sit at room temperature overnight. The next day, the bones were placed in a 37°C radiant heat oven for four days. Once polymerization was completed, glass vials were removed from the oven, incubated at -20°C for one hour and glass was removed by breaking.

Specimen blocks were then trimmed and sanded on a Buehler Mataserve Grinder-Polisher (Buehler, Lake Bluff, IL) with 60 or 240 grit grinding paper (Carobinmet Special Silicon Carbide Grinding Paper, Buehler) for course grinding and polishing,
respective. Prepared bone blocks were clamped directly into Reichert-Jung Polycut E Microtome block holder, and trimmed using a tungsten-carbide knife, D-profile (Delaware Diamond Knives, Wilmington, DE). The block face and knife were moistened with 40% EtOH to facilitate sectioning. Once the area of the ulnar bone was reached, 7 µm sections were carefully removed from the knife blade with fine forceps. Sections were placed on a 95% ethanol that was on chrome-alum-gel coated slides, and teased and flattened using a very fine paintbrush. Sections were covered with a strip of clear plastic and remaining wrinkles and excess ethanol were removed by rolling over the plastic with a small roller. Slides were stacked and pressure was applied using a paper clamp. Sections mounted on chrome-alum-gel coated slides were incubated overnight at 37°C to adhere sections to slide. The sections for VonKossa staining were mounted on the Silane-Plus coated slides, and sections for Trichrome and H&E staining were mounted on charged slides. Sections were adhered to the slides by overnight incubation at 60°C.

7.14.3. Histological Staining

7.14.3.1. Hematoxylin & Eosin Stain

7.14.3.1.1. Materials
- For paraffin-embedded sections: Xylene/Histoclear
- For sMMA-embedded sections: Cellsolve (ethylene glycol monomethyl ether acetate, Fisher Scientific)
- Ethanol (100% and 95%)
- dH₂O
- Hematoxylin (Fisher, #67-650-09)
- Bluing Solution (Fisher, #67-690-02)
- Eosin (Fisher, #23-245-658)
- Mounting Media (Thermo Scientific, #22-110-610)

### 7.14.3.1.2. Protocol

<table>
<thead>
<tr>
<th>Solution</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xylene/Histoclear/Cellsolve</td>
<td>25 min</td>
</tr>
<tr>
<td>Xylene/Histoclear/Cellsolve</td>
<td>25 min</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>5 min</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>3 min</td>
</tr>
<tr>
<td>95% ethanol</td>
<td>5 min</td>
</tr>
<tr>
<td>95% ethanol</td>
<td>3 min</td>
</tr>
<tr>
<td>dH₂O</td>
<td>2 min</td>
</tr>
<tr>
<td>Hematoxylin</td>
<td>1 min</td>
</tr>
<tr>
<td>Running tap H₂O</td>
<td>Until clear</td>
</tr>
<tr>
<td>Bluing Solution</td>
<td>1 min</td>
</tr>
<tr>
<td>Running tap H₂O</td>
<td>Till clear</td>
</tr>
<tr>
<td></td>
<td>Time</td>
</tr>
<tr>
<td>----------------</td>
<td>--------</td>
</tr>
<tr>
<td>dH₂O</td>
<td>2 min</td>
</tr>
<tr>
<td>95% Ethanol</td>
<td>2 min</td>
</tr>
<tr>
<td>Eosin</td>
<td>30 sec</td>
</tr>
<tr>
<td>dH₂O</td>
<td>2 min</td>
</tr>
<tr>
<td>95% ethanol</td>
<td>3 min</td>
</tr>
<tr>
<td>95% ethanol</td>
<td>5 min</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>3 min</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>5 min</td>
</tr>
<tr>
<td>Xylene/Histoclear</td>
<td>3 min</td>
</tr>
<tr>
<td>Xylene/Histoclear</td>
<td>5 min</td>
</tr>
<tr>
<td>Mounting Media</td>
<td>Coverslip</td>
</tr>
</tbody>
</table>

7.14.3.1.3. Results

- Nuclei ------------------------- blue
- Cytoplasm ------------------------ pink

7.14.3.2. Masson’s Tri-Chrome Stain

7.14.3.2.1. Materials
- For paraffin-embedded sections: Xylene/Histoclear
- For sMMA-embedded sections: Cellsolve (ethylene glycol monomethyl ether acetate, Fisher Scientific)
- Ethanol (100% and 95%)
- dH₂O
- Masson’s Trichrome Staining Kit (Sigma-Aldrich #HT15)
- Biebrich Scarlet-Acid Fuchsin Solution (0.9%)
- Phosphotungstic Acid Solution (10%)
- Phosphomolybdic Acid Solution (10%)

  - Prepare Working Phosphotungstic/Phosphomolybdic Acid Solution by mixing 1 volume of Phosphotungstic Acid Solution, and 1 volume Phosphomolybdic Acid Solution, with 2 volumes of deionized water.

  - Discard after one use.

- Aniline Blue Solution (2%)
  - Bouin’s Solution (Sigma-Aldrich, # HT10-1-32)
  - Weigert’s Iron Hematoxylin solution
  - 1% Acetic Acid
  - Mounting Media (Thermo Scientific, #22-110-610)

### 7.14.3.2.2. Protocol

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xylene/Histoclear/Cellsolve</td>
<td>25 min</td>
</tr>
<tr>
<td>Xylene/Histoclear/Cellsolve</td>
<td>25 min</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>5 min</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>3 min</td>
</tr>
<tr>
<td>95% ethanol</td>
<td>5 min</td>
</tr>
<tr>
<td>95% ethanol</td>
<td>3 min</td>
</tr>
<tr>
<td>dH2O</td>
<td>2 min</td>
</tr>
<tr>
<td>Stain/Chemical Name</td>
<td>Time/Condition</td>
</tr>
<tr>
<td>-------------------------------------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>Bouin’s Solution</td>
<td>15 min, 56°C</td>
</tr>
<tr>
<td>Running tap H₂O</td>
<td>Until clear</td>
</tr>
<tr>
<td>Weigert’s Iron Hematoxylin Solution</td>
<td>5 min</td>
</tr>
<tr>
<td>Running tap H₂O</td>
<td>Till clear</td>
</tr>
<tr>
<td>dH₂O</td>
<td>2 min</td>
</tr>
<tr>
<td>Biebrich Scarlet-Acid Fucshin</td>
<td>5 min</td>
</tr>
<tr>
<td>dH₂O</td>
<td>2 min</td>
</tr>
<tr>
<td>Phosphotungstic/Phosphomolybdic Acid</td>
<td>5 min</td>
</tr>
<tr>
<td>Solution</td>
<td></td>
</tr>
<tr>
<td>Aniline Blue Solution</td>
<td>5 min</td>
</tr>
<tr>
<td>Acetic Acid</td>
<td>2 min</td>
</tr>
<tr>
<td>dH₂O</td>
<td>2 min</td>
</tr>
<tr>
<td>95% ethanol</td>
<td>3 min</td>
</tr>
<tr>
<td>95% ethanol</td>
<td>5 min</td>
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<tr>
<td>100% ethanol</td>
<td>3 min</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>5 min</td>
</tr>
<tr>
<td>Xylene/Histoclear</td>
<td>3 min</td>
</tr>
<tr>
<td>Xylene/Histoclear</td>
<td>5 min</td>
</tr>
<tr>
<td>Mounting Media</td>
<td>Coverslip</td>
</tr>
</tbody>
</table>

* Unless indicated otherwise, the step was performed at room temperature.
7.14.3.2.3. Results
- Collagen fibers------------------------ blue
- Nuclei----------------------------- black
- Cytoplasm, fibrin, erythrocytes ------- red

7.14.3.3. Von Kossa Stain
7.14.3.3.1. Materials
- For paraffin-embedded sections: Xylene/Histoclear
- For sMMA-embedded sections: Cellsolve (ethylene glycol monomethyl ether acetate, Fisher Scientific)
- 1% Aqueous Silver Nitrate Solution
- 5% Sodium Carbonate-Formaldehyde
- 0.1% Nuclear Fast Red Solution
- Ethanol (100% and 95%)
- dH₂O
- Mounting Media (Thermo Scientific, #22-110-610)

7.14.3.3.2. Protocol

<table>
<thead>
<tr>
<th>Solution</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xylene/Histoclear/Cellsolve</td>
<td>25 min</td>
</tr>
<tr>
<td>Xylene/Histoclear/Cellsolve</td>
<td>25 min</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>5 min</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>3 min</td>
</tr>
<tr>
<td>Process</td>
<td>Duration</td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>----------</td>
</tr>
<tr>
<td>95% ethanol</td>
<td>5 min</td>
</tr>
<tr>
<td>95% ethanol</td>
<td>3 min</td>
</tr>
<tr>
<td>dH₂O</td>
<td>2 min</td>
</tr>
<tr>
<td>1% silver nitrate solution</td>
<td>30 min; dark</td>
</tr>
<tr>
<td>Running tap H₂O</td>
<td>Until clear</td>
</tr>
<tr>
<td>5% sodium carbonate-formaldehyde</td>
<td>2 min</td>
</tr>
<tr>
<td>Running tap H₂O</td>
<td>Till clear</td>
</tr>
<tr>
<td>dH₂O</td>
<td>2 min</td>
</tr>
<tr>
<td>Methyl green pyronin</td>
<td>20 min</td>
</tr>
<tr>
<td>dH₂O</td>
<td>2 min</td>
</tr>
<tr>
<td>95% ethanol</td>
<td>3 min</td>
</tr>
<tr>
<td>95% ethanol</td>
<td>5 min</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>3 min</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>5 min</td>
</tr>
<tr>
<td>Xylene/Histoclear</td>
<td>3 min</td>
</tr>
<tr>
<td>Xylene/Histoclear</td>
<td>5 min</td>
</tr>
<tr>
<td>Mounting Media</td>
<td>Coverslip</td>
</tr>
</tbody>
</table>

**7.14.3.3. Results**
- Calcium salts --------- black or brown-black
- Osteoid ----------------------red or light pink
7.15. Immunohistochemistry

7.15.1. Materials

- Histoclear or Xylene
- Ethanol (100%, 95%)
- Distilled water
- Dako Target retrieval (S1700, Dako)
- Hydrogen Peroxide (216763, Sigma-Aldrich)
- Phosphate buffered saline 1X (Sigma)
- Bovine Serum Albumin (BSA) (A9418-5G, Sigma-Aldrich)
- Primary Antibody
- Secondary Antibody (HRP-conjugated or fluorescent)
- Normal Goat Serum (NGS) (S-1000, Vector Laboratories)
- Blocking Solution (10 mL PBS, 0.05 g BSA)
- Primary/Secondary Antibody Solution (10 mL PBS, 0.05 g BSA, 300 ul Normal Goat Serum, and 1° Antibody)
- TBS plus 0.025% Triton X-100
- DAB Substrate Kit, 3,3’-diaminobenzidine (SK-4100, Vector)
- PAP pen (#ab2601, Abcam)
- Mounting Media (Thermo Scientific, #22-110-610)
7.15.2. Protocol

Immunohistochemistry is the localization of antigens or proteins in tissue sections by the use of labeled antibodies as specific reagents through antigen-antibody interactions that are visualized by a marker such as fluorescent dye or enzyme. Before proceeding with the staining protocol, the slides were deparaffinized and rehydrated, since incomplete removal of paraffin can cause poor staining of the section. The slides were placed in Histoclear for 5 minutes, and this was repeated two times. The slides then underwent serial dehydration. The slides were placed in 100% ethanol for 10 minutes (2 times), and then 95% ethanol for 10 minutes (2 times). The slides were transferred to distilled water for 5 minutes (2 times), and then antigen retrieval (Dako Target retrieval) was performed for 5 minutes at 98°C. This step serves to break the methylene bridges and expose the antigenic sites in order to allow the antibodies to bind. The slides were then washed 2 times for 5 minutes each in TBS and 0.025% Triton X-100 with gentle agitation. The slides were blocked in 10% normal serum with 1% BSA in TBS for 2 hours at room temperature. The slides were then drained for a few seconds (do not rinse) and then wiped around the sections with tissue paper. The primary antibody diluted in TBS with 1% BSA was applied, and incubated overnight at 4°C. The slides were rinsed 2 times for 5 minutes each in TBS and 0.025% Triton with gentle agitation. If an HRP conjugate was used for detection, the slides were incubated in 0.3% H₂O₂ in TBS for 15 minutes. For enzymatic detection
(HRP conjugates), HRP-conjugated secondary antibody was applied to the slide diluted to the concentration recommended by the manufacturer in TBS with 1% BSA, and incubated for 1 hour at room temperature. For fluorescent detection, fluorophore-conjugated secondary antibody was applied to the slide diluted to the concentration recommended by the manufacturer in TBS with 1% BSA, and incubated for 1 hour at room temperature. The slides were rinsed 3 times for 5 minutes each with TBS. If using fluorescent detection, the procedure ended at this step and the slides were coverslipped with mounting medium. If visualizing the protein with a chromogen, the slides were developed with a chromogen (i.e., 3,3'- Diaminobenzidine (DAB)) for 10 minutes at room temperature. The slides were rinsed in running tap water for 5 minutes, and then counterstained with hematoxylin. The slides were then dehydrated, cleared and mounted with coverslips.

7.16. **Immunocytochemistry**

7.16.1. **Materials**

7.16.2. **Histoclear or Xylene**

- Ethanol (100%, 95%)
- Distilled water
- Dako Target retrieval (S1700, Dako)
- Hydrogen Peroxide (216763, Sigma)
- Phosphate buffered saline 1X (Sigma)
- Bovine Serum Albumin (BSA) (A9418-5G, Sigma)
• Primary Antibody
• Secondary Antibody (HRP-conjugated or fluorescent)
• Normal Goat Serum (S-1000, Vector Laboratories)
• Blocking Solution (10 mL PBS, 0.05 g BSA)
• Primary/Secondary Antibody Solution (10 mL PBS, 0.05 g BSA, 300 ul Normal Goat Serum, and 1° Antibody)
• TBS plus 0.025% Triton X-100
• DAB Substrate Kit, 3,3’-diaminobenzidine (SK-4100, Vector)
• PAP pen (#ab2601, Abcam)
• Mounting Media (Thermo Scientific, #22-110-610)
• Coverslips

7.16.3. Protocol
To examine cell expression of specific proteins, we performed immunocytochemistry. First, cells were removed from cell culture, washed briefly with PBS, and then covered to a depth of 2-3 mm with 2-4% formaldehyde in PBS for 15 minutes at room temperature. The fixative was aspirated, and then the cells were rinsed three times in PBS for 5 minutes each. To permeabilize the cells, the cells were covered with ice-cold 100% methanol, and incubated in methanol for 10 minutes at –20°C. The specimens were then incubated with 10% normal blocking serum in PBS for 20 minutes to suppress non-specific binding of IgG. Blocking serum ideally should be
derived from the same species in which the secondary antibody is raised. The specimens were then incubated with primary antibody for 60 minutes. Optimal antibody concentration should be determined by titration; recommended range is 0.5–5.0 µg/ml in PBS with 1.5% normal blocking serum. The specimens were then washed with three changes of PBS for 5 minutes each, and then incubated for 45 minutes with fluorochrome-conjugated secondary antibody diluted to 1–5 µg/ml in PBS with 1.5%–3% normal blocking serum. The specimens were then washed with three changes of PBS, and incubated with DAPI to stain the nuclei. The cells were then covered in multi-well plate with aqueous mounting medium, and viewed under a fluorescence microscope.

### 7.17. Live/Dead Cell Viability Analysis

#### 7.17.1. Materials

- Phosphate buffered saline 1X (Sigma)
- Live/Dead® Viability/Cytotoxicity Kit (#L3224, Invitrogen)
- Glass Bottom Dishes, 35 mm uncoated (P35G-1.0-14-C, MatTek)

#### 7.17.2. Protocol

To examine cell viability, we utilized the Live/Dead Viability Kit, which contains calcein AM and ethidium homodimer-1. Membrane-permeant calcein AM is cleaved by esterases in live cells to yield cytoplasmic green
fluorescence, and membrane-impermeant ethidium homodimer-1 labels nucleic acids of membrane-compromised cells with red fluorescence. The sample was rinsed with PBS two times, and incubated with Live/Dead solution (10 milliliters PBS, 4 µl ethidium bromide, 1.25 µl calcien AM) for 1 hour. The samples were rinsed with once with PBS, and viewed under fluorescent microscope or confocal microscope (requires sample to be on Glass Bottom Dishes).

7.18. Gene Expression Analysis

7.18.1. RNA Extraction

7.18.1.1. Materials

• Qiagen RNeasy Mini Kit
  o RNeasy Mini Spin Columns (pink)
  o Collection Tubes (1.5 ml)
  o Collection Tubes (2 ml)
  o Buffer RLT (Add 10 µl β-ME per 1 ml Buffer RLT)
  o Buffer RW1
  o Buffer RPE (concentrate; add 4 volumes of ethanol (96–100%) as indicated on the bottle to obtain a working solution.)
  o RNase-Free Water

• QIAshredder Spin Columns
• 14.3 M β-mercaptoethanol (β-ME)
• Sterile, RNase-free pipette tips
• 96–100% ethanol

7.18.1.2. **Protocol**

We used Qiagen RNeasy Mini Kit to extract and purify RNA from cells cultured on scaffolds. First, to directly lyse cells, 0.5 milliliters of Buffer RLT was added, and then pipetted the lysate directly into a QIAshredder spin column placed in a 2 ml collection tube, and centrifuged for 2 min at full speed. 1 volume of 70% ethanol was added to the homogenized lysate, mixed well by pipetting, transferred to an RNeasy spin column placed in a 2 ml collection tube, and centrifuged for 15 seconds at ≥8000 x g (≥10000 RPM). The flow-through was discarded. If the sample volume exceeded 700 µl, successive aliquots was centrifuged in the same RNeasy spin column. The flow-through was discarded after each centrifugation, and 700 µl Buffer RW1 was added to the RNeasy spin column and then centrifuged for 15 seconds at ≥8000 x g (≥10000 rpm RPM) to wash the spin column membrane. The flow-through was discarded, and 500 µl Buffer RPE was added to the RNeasy spin column, and then centrifuged for 15 seconds at ≥8000 x g (≥10000 RPM) to wash the spin column membrane. The flow-through was discarded, and 500 µl Buffer RPE was added to the RNeasy spin column, and centrifuged for 2 minutes at ≥8000 x g (≥10000 RPM) to wash the spin column
membrane. The RNeasy spin column was placed in a new 2 ml collection tube, and centrifuged at full speed for 1 minute. The RNeasy spin column was placed in a new 1.5 ml collection tube, and 30–50 µl RNase-free water was added directly to the spin column membrane, and centrifuged for 1 minute at ≥8000 x g (≥10000 RPM) to elute the RNA. We used the NanoDrop machine to measure the RNA concentration in each sample. 3 µg of RNA per sample was used to carry out gene quantification analysis.

7.19. Quantitative Polymerase Chain Reaction (Q-PCR)

7.19.1. Materials

- Clontech Sprint RT Complete cDNA synthesis kit (Clontech; Mountain View, CA)
- BioRad MyiQ2 Two-Color Real-Time PCR Detection System
- 2x iQ real-time PCR Supermix (BioRad; Hercules, CA)
- TaqMan® Gene Expression Assay Probes (Applied Biosystems; Carlsbad, CA)
- Microseal 96-Well PCR Plates (BioRad; Hercules, CA)
- iQ real-time PCR Supermix
- TaqMan® Gene Expression Assay probe (Applied Biosystems)
7.19.2. Protocol

For cDNA synthesis, 2 µg total RNA was used as a template for Clontech Sprint RT Complete cDNA synthesis kit (Clontech; Mountain View, CA) in a total volume of 20 µl. For quantitative real time PCR, BioRad MyiQ2 Two-Color Real-Time PCR Detection System, 2x iQ real-time PCR Supermix (BioRad; Hercules, CA), TaqMan® Gene Expression Assay Probes (Applied Biosystems; Carlsbad, CA) were loaded in Microseal 96-Well PCR Plates (BioRad; Hercules, CA). Each well contained 10 µl of iQ real-time PCR Supermix, 1 ul of TaqMan® Gene Expression Assay probe and 9 µl of diluted cDNA. Threshold cycle values of target genes was standardized against GAPDH expression and normalized to the expression in the control culture. The –fold change in expression was calculated using the ΔΔCt comparative threshold cycle method.

7.20. Angiogenic Potential Assays

7.20.1. 2D Matrigel Assay

7.20.1.1. Materials

- BD Matrigel Basement Membrane Matrix High Concentration (354248, BD Biosciences)
- Serum-free DMEM
- Endothelial Growth Media-2 (EGM-2) (Lonza)
- Glass Bottom Dishes, 35 mm uncoated (P35G-1.0-14-C, MatTek)
- Live/Dead® Viability/Cytotoxicity Kit (#L3224, Invitrogen)
- Phosphate buffered saline 1X (Sigma)

7.20.1.2. Protocol
To evaluate the angiogenic potential of endothelial cells, we cultured the cells on Matrigel, a solid gel of basement proteins prepared from the Engelbreth Holm-Swarm (EHS) mouse tumor. Cells with high angiogenic potential will rapidly align and form hollow tube-like structures. For this, we diluted the Matrigel (stock concentration 20 mg/ml) to 10 mg/ml with serum-free DMEM, and evenly coated glass bottom 35 mm cell culture dishes with 75 µl of diluted Matrigel, and incubate for 30 minutes at 37°C. 300 µl cell suspension containing 80,000 cells was plated, and incubated for 5-6 hours. The cells were stained with Live/Dead staining, and imaged via confocal microscopy. The following criteria were quantified: pattern recognition, branch point counting, average and total capillary tube length. For pattern recognition quantification, we assigned numerical values for specific visual patterns (see below) (adapted from Millipore, In Vitro Angiogenesis Assay Kit).

<table>
<thead>
<tr>
<th>Pattern</th>
<th>Value</th>
</tr>
</thead>
</table>

208
### 7.20.2. 3D Matrigel Assay

#### 7.20.2.1. Materials
- BD Matrigel Basement Membrane Matrix High Concentration (354248, BD Biosciences)
- 48 well cell culture plate
- Cell culture media

#### 7.20.2.2. Protocol
Matrigel was thawed overnight at 4°C on ice. Matrigel was handled with pre-cooled pipettes, tips, and tubes when preparing for useage, since Matrigel will gel rapidly at 22°C to 35°C. Cells were encapsulated in Matrigel (10^6 cells/mL Matrigel) by mixing a 1x10^6 cell suspension in chilled media (750 µl) with 750 µl of Matrigel (20 mg/ml), to make a final concentration of 10 mg/ml of Matrigel. 500 µl of this Matrigel/cell suspension mixture was seeded into each well in a 48-well plate. 3 samples per group were seeded, and cultured in a 1-
to-1 mixture of osteogenic and angiogenic medium for 7 days in 48 well cell culture plates. The following twelve sample groups were studied: PB-EPCs alone; BM-EPCs alone; MSCs:PB-EPCs co-culture ratios of 1:4, 1:2, 1:1, 2:1, and 4:1; and MSCs:BM-EPCs co-culture ratios of 1:4, 1:2, 1:1, 2:1, and 4:1. Three-dimensional samples (10 mm diameter, 2 mm height) were paraffin-embedded, sectioned and then stained with hematoxylin and eosin. Four samples per group were embedded, and three sections per sample were stained (one from top, middle and bottom; approximately 200 µm apart from each other). Stained sections were analyzed under the light microscope Olympus BX50 with Olympus DP70 camera. Twelve images viewed under 10X magnification, and the number of branches were counted for each sample.

7.21. Flow Cytometry

7.21.1. Materials

- 1x10^6 cell suspension (for each marker of interest)
- BD Cytofix/Cytoperm™ BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit (#554714, BD Bioscience)
- BD Pharmingen™ Stain Buffer (FBS) (#554656, BD Bioscience)
• BD Perm/Wash\textsuperscript{TM} buffer (#554723, BD Bioscience)
• Live/Dead Fixable Stain in Far Red (#L10120, Invitrogen)
• Primary antibody
• Fluorochrome-conjugated secondary antibody

7.21.2. Protocol

Flow cytometry (FCM) is a technique that may be used for counting and examining surface and intracellular proteins on cells.

7.21.2.1. Surface Staining Cells

To examine one cell surface marker, a sample of cells in suspension containing at least $1 \times 10^6$ cells was centrifuged. The supernatant was discarded, and the cells were washed once with 1 milliliter of Staining Buffer, and the resuspended in 1 milliliter of Staining Buffer containing 1 µL of the reconstituted fluorescent reactive dye. The suspension was incubated at room temperature or on ice for 30 minutes, protected from light, and then washed with 1 milliliter of Staining Buffer and resuspended in 50 µl of Staining Buffer. The cell suspension of $\sim 10^6$ cells in 50 µl of Staining Buffer was then stained with the appropriate amount of a primary antibody specific for 30 minutes at 4°C. The excess antibody was washed off following staining, and 1.5-2 milliliters of Staining buffer was added to each tube, and centrifuged 5 minutes at 2000 RPM. The supernatant was aspirated, and 100 µl of staining buffer was added to each tube, and then 0.5-1 µg of the fluorochrome-conjugated secondary antibody. The
sample was vortexed and incubated for 15-30 minutes in a covered ice bucket. To wash off excess antibody following staining, 1.5-2 milliliters of staining buffer was added to each tube and then centrifuged in tabletop microfuge for 5 minutes at 2000 RPM. The supernatant was aspirated off, and the cells were resuspended in 250 µl for tubes of Fixation/Permeabilization solution for 20 min. at 4°C. (Note: Cell aggregation is avoided by vortexing prior to the addition of the Fixation/Permeabilization solution.) The cells were washed two times in 1× BD Perm/Wash™ buffer (e.g., 1 milliliters /wash for staining in tubes) and pellet. (NOTE: BD Perm/Wash™ buffer must be maintained in washing steps to keep cells permeabilized.) Resuspend in Staining Buffer prior to flow cytometric analysis.

7.21.2.2. Intracellular Staining Cells
A sample of cells in suspension (> 1 × 10⁶ cells) was centrifuged, and the supernatant was discarded. The cells were washed once with 1 milliliter of Staining Buffer. The cells were resuspended in 1 milliliters of Staining Buffer containing 1 µL of the reconstituted fluorescent reactive dye, and then incubated at room temperature or on ice for 30 minutes, protected from light. The cells were washed with 1 milliliters of Staining Buffer, and then resuspended in 250 µl Fixation/Permeabilization solution for 20 minutes at 4°C. The cells were washed two times in 1× BD Perm/WashTM buffer (e.g., 1 milliliters/wash for staining in tubes) and pellet. (NOTE: BD
Perm/WashTM buffer must be maintained in washing steps to keep cells permeabilized.) The cells were resuspended in 50 µl of BD Perm/WashTM Buffer, and then stained the ~10⁶ cells in 50 µl of BD Perm/WashTM buffer with the appropriate amount of a primary antibody specific for 30 min at 4°C. To wash off excess antibody following staining, 1.5-2 milliliters of BD Perm/WashTM buffer was added to each tube, and then centrifuged for 5 minutes at 3000-4000 RPM for intracellular staining. The supernatant was aspirated off, and 100 µl of BD Perm/WashTM buffer was added to each tube with 0.5-1 µg fluorochrome-conjugated secondary antibody. The sample was vortexed and incubated for 15-30 minutes in a covered ice bucket. To wash off excess antibody following staining, 1.5-2 milliliters of BD Perm/WashTM buffer was added to each tube, and centrifuged in a tabletop microfuge for 5 minutes at 2000 RPM (or 3000-4000 RPM for intracellular staining). The sample was then resuspended in 400 µl Staining Buffer prior to flow cytometric analysis.

7.22. Western Blot Analysis

7.22.1. Materials

- CellLytic M buffer and Protease Inhibitor (Sigma)
- 4-15% Tris-HCl Ready Gels
- Laemmli Sample Buffer
- Mini-PROTEAN Tetra System
7.22.2. Protocol

CellLytic M buffer and Protease Inhibitor (Sigma; St. Louis, MO) were added to cells and then incubated at 4°C for 30 minutes. Cells were removed by mechanical scrapping and spun down at 10,000 rpm for 5 minutes. Protein concentrations were measured using BCA Protein Assay Kit and the absorbance of the samples were measured at 562 nm after 30 minute incubation at 37°C. Each sample was prepared with Laemmli Sample Buffer and samples were boiled for 5 minutes. 25 µg of each sample were run on 4-15% Tris-HCl Ready Gels for western blot protein electrophoresis. Mini-PROTEAN Tetra System, 10x Tris/Glycine/SDS Buffer, Precision Plus
Protein Dual Color Standard were used and gels were run at a constant 100 volts. Gels were transferred at a constant 100 Volts for 2 hours using 10x Tris/Glycine Buffer, Mini Trans-Blot Electrophoresis Transfer Cell, Blot Papers and 0.2 μm Nitrocellulose Membrane. Membranes were blocked for 2 hours at 4°C in 10% milk/TBS-T solution (10x Tris-Buffered Saline, 0.1% Tween-20). Membranes were washed with TBS-T solution after each incubation. Membranes were incubated overnight with primary antibodies diluted in 5% milk/TBS-T solution at 4°C. Membranes were incubated for 45 minutes with secondary antibody diluted in 5% milk/TBS-T solution at 4°C and then washed three times with TBS-T. Super Signal West Pico Chemiluminescent Substrate was used for detection, and CL-XPosure Film was used for exposure of the membranes. All reagents and materials for western blot analysis were purchased from BioRad (Hercules, CA) unless otherwise indicated.

7.23. Animal Models

7.23.1. SCID Mouse Subcutaneous Implant Model

Fox Chase CB17 SCID® (Severe Combined Immunodeficiency) male mice (Charles River Laboratories, Cambridge, MA), ages 50–56 days were used for the mouse subcutaneous implant model. Prior to surgery, animals were given 0.05-0.1 mg/kg Buprenorphine subcutaneous for pre-operative analgesia. Animals were anesthetized with an intraperitonial injection of a mixture of ketamine (90-120 mg/kg body wt) and xylazine (5-10 mg/kg body
wt) given IP. After the animals were fully anesthetized, the surgical site on animals (dorsum) was shaved and cleaned with 70% ethanol, betadine and again with 70% ethanol. Two subcutaneous pouches were created bilaterally of the dorsal side (i.e., both the sides of the spine) by making incisions approximately 2.5 cm long using blunt dissection techniques, and a sterile polymer scaffold will be inserted into each pouch.

Post-surgery, animals were kept in recovery cages placed on heating pads and under warm light to maintain body temperature, which was monitored with a rectal thermometer every 15-20 minutes until recovery. The animals were administered Buprenorphine (0.05-0.1 mg/kg SQ) twice per day 8-10 hours apart for up to 48 hours and then as needed to minimize pain post surgery. The following signs of pain were monitored: lack of grooming, sitting haunched up in a corner of the cage, rapid and shallow breathing, reaching less frequently for food and water. Furthermore, the surgical wounds were evaluated daily during the first postoperative week for the presence of infection or dehiscence. At pre-determined time-points, animals were sacrificed by CO₂ narcosis followed by cervical dislocation.

7.23.2. New Zealand White Rabbit Ulnar Critical-Size Bone defect Model

New Zealand White male rabbits (4-5 kg) from Millbrook Breeding Labs (Amherst, MA, USA) were used to perform the rabbit ulnar critical-size bone defect model. Animals were housed with ad libitum access to food and water prior to surgery. A dose of 0.01-0.05 mg/kg buprenorphine was given IM
approximately 1 hour prior to, 5-10 mg/kg Xylazine given IM 10 min prior to, and 33 – 35 mg/kg Ketamine IM. Sedation was confirmed through ear/toe pinch. Sedation was maintained during surgery with 0.5-2% isoflurane.

Sterile surgical technique and sterile fields was maintained at all times prior to and during surgery. Briefly, protective lubricant was applied to eyes of the rabbit, and an ear catheter was placed for administration of IV fluids (i.e., Normosol R, Lactated Ringers) during anesthesia at approximately 10ml/kg/hr. Rabbits were then intubated. Once intubated, isoflurane 0.5-2% was initiated and maintained through the surgical procedure. The surgical site on the animal was shaved, cleaned with 70% ethanol, betadine, and 70% ethanol again, and then placed on a sterile drape and the area around the surgical site covered with sterile drapes. The surgeon wore a sterile gown, and gloves and will wear a face mask, eye shield, and cap. The instruments were autoclaved at 121°C for 15 minutes to ensure sterility. Instruments were opened within the sterile field. Between surgeries on different animals, unused, sterile instruments were used. A surgical hypothermia unit that uses circulating warm water was placed under the rabbit during surgery.

Under sterile conditions, a lateral incision approximately 2.5 centimeters long was made and the tissue overlying the ulna was dissected. A 1.5 cm segmental osteoperiosteal defect was created in the middle of the ulna using an oscillating saw. The defect was filled with the construct, and the soft tissues was closed in layers, using resorbable sutures to close muscle tissue. The skin was approximated and closed using 3-0 absorbable sutures.
Subcuticular suturing with buried knots will be employed to minimize or prevent chewing of the surgical area, along with surgical glue. The overlying skin was also be closed with non-degradable sutures.

After the surgical procedure was complete, animals were kept in the recovery room. Pain will be monitored through frequent observations: grinding of teeth, lack of grooming, sitting hunched up in a corner of the cage, rapid and shallow breathing, reaching less frequently for food and water. When the animal was ready to be extubated, a 25mcg fentanyl patch was applied to a shaved area over the dorsal neck/interscapular region. The patch was maintained at therapeutic levels for 72 hours, at which time it was either be removed or replaced. If there was an unexpected time lapse, then another dose of buprenex was administered to fill the gap until patch fentanyl is absorbed (which may take up to 8 hours). All animals were weighed once per week. Animals were housed with access to food and water ad libitum. Post op analgesia was considered for up to 5-6 days if needed. Animals were given hay cubes, bunny blocks, and fresh produce to stimulate appetite or for environmental enrichment.
8. APPENDIX 2 - List Of Publications

8.1. RESEARCH PAPERS:


8.2. REVIEW ARTICLES:


### 8.3. BOOK CHAPTERS:


### 8.4. ABSTRACTS:


Igwe J, Amini AR, Nukavarapu SP. Fabrication and Evaluation of a Novel Scaffold System with High-Density Cell Seeding for Bone Regeneration: An


9. REFERENCES

159. Fuchs S, Motta A, Migliaresi C, Kirkpatrick CJ. Outgrowth endothelial cells isolated and expanded from human peripheral blood progenitor cells as a potential source of autologous cells for endothelialization of silk fibroin biomaterials. Biomaterials. 2006;27(31):5399-408.


