Characterization of Adult Progenitor Cells and their Fate in a Rat Femur Fracture Model

by

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A thesis submitted in conformity with the requirements for the degree of Master’s of Science
Institute of Medical Science
University of Toronto

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Abstract

This study was carried out to characterize two progenitor cell populations in the context of orthopaedics and to examining their fate in a rat bone defect model. Endothelial progenitor cells (EPCs) and mesenchymal stem cells (MSCs) isolated from syngeneic bone marrow were characterized utilizing flow cytometry and osteogenic/angiogenic differentiation assays in vitro. To assess in vivo fate, genetically labeled cells were implanted into a defect and examined at two and four weeks post-implantation. Fluorescent microangiography (FMA) was used to model nascent vasculature two weeks post-implantation. The results show populations that resemble established definitions. In vitro, EPCs demonstrated a similar osteogenic capacity to MSCs and superior angiogenic capacity on the basis of tube formation. In vivo experiments showed presence of cells at the fracture site at 2 and 4 weeks. EPCs showed a trend towards increased vessel formation with FMA. These results provide groundwork for future investigation into mechanisms of healing.
Acknowledgments

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Contributions

Contributions to this project were made by a number of people. Sarah Ketchenson contributed to the execution of numerous protocols of this project in addition to data collection. Dr. Aaron Nauth performed all animal surgeries. Tony Lin contributed to AcLDL/Lectin data. Flow cytometry and histology equipment/training were provided by Li Ka Shing Knowledge Institute core facilities. FMA protocol and technical help was provided by Michael Kuliszewski of the Dr. Leong-Poi lab. Immunohistochemistry was performed by the pathology core at The Toronto Centre for Phenogenomics.
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<tbody>
<tr>
<td>AICBG</td>
<td>Autogeneous Iliac Crest Bone Graft</td>
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<td>ALK-2</td>
<td>Alkaline Phosphatase-2</td>
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<td>ANG-1</td>
<td>Angiopoietin-1</td>
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<td>BMP</td>
<td>Bone Morphogenic Protein</td>
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<td>CBT</td>
<td>Cell-Base Therapy</td>
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<td>eMSC</td>
<td>Endothelial-Mesenchymal Stem Cell</td>
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<td>EndMT</td>
<td>Endothelial to Mesenchymal Transition</td>
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<td>EO</td>
<td>Endochondral Ossification</td>
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<td>EPC</td>
<td>Endothelial Progenitor Cell</td>
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<td>FGF</td>
<td>Fibroblast Growth Factor</td>
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<td>FMA</td>
<td>Fluorescent Microangiography</td>
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<td>FOP</td>
<td>Fibrodysplasia Ossificans Progressiva</td>
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<td>FSC-A</td>
<td>Forward Scatter-Area</td>
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<td>Abbreviation</td>
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<td>FSC-H</td>
<td>Forward-Scatter-Height</td>
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<tr>
<td>G-CSF</td>
<td>Granulocyte-Colony Stimulating Factor</td>
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<td>GFP</td>
<td>Green Fluorescent Protein</td>
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<tr>
<td>HAC</td>
<td>Hydroxyapatiteceramic</td>
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<td>HGF</td>
<td>Hepatocyte Growth Factor</td>
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<td>IGF</td>
<td>Insulin-like Growth Factor</td>
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<td>IHC</td>
<td>Immunohistochemistry</td>
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<td>IO</td>
<td>Intramembranous Ossification</td>
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<td>MSC</td>
<td>Mesenchymal Stem Cell</td>
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<tr>
<td>NSAIDs</td>
<td>Non-Steroidal Anti-inflammatory Drugs</td>
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<td>PDGF</td>
<td>Platelet Derived Growth Factor</td>
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<td>TGFβ-2</td>
<td>Transforming Growth Factor</td>
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<td>Vascular Endothelial Growth Factor</td>
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1 Literature Review

1.1 Clinical Background

The treatment and management of bone defects remains a significant clinical challenge for orthopaedic surgeons. Although our understanding of bone biology and the process of fracture healing has rapidly increased over the last couple decades, few novel therapies have been introduced to effectively enhance fracture healing. The current management techniques available for bone defects remain sub-optimal, often leading to multiple surgical procedures, long hospital stays, and extended periods of non-weight bearing activity. Difficulties with treatment can increase patient morbidity, cause disability, and in extreme cases, may lead to a complete amputation of the affected limb. A novel therapy that optimizes healing while decreasing disability time would benefit individual patients and reduce the associated socioeconomic burden of fracture care.

One persistent problem within the orthopaedic field is a situation where a bone defect fails to heal, forming what is termed as a nonunion. This failure to heal has been reported to occur in approximately 10% of all long bone fractures and up to 13% of tibia fractures (Audige et al. 2005; Rodriguez-Merchan and Forriol 2004). There are a number of factors that can significantly increase the odds of nonunion formation on a patient level. These include increased age, nutrition status, complexity of the fracture, smoking status, and the use of NSAIDs (Calori et al. 2007). The scale of economic impact is reflected in statistics
from the United States where musculoskeletal conditions are reported to represent $126 billion in health care costs, affecting 6.8 million patients (Praemer, Furner, and Rice 1992).

Bone defects occur when patients undergo severe skeletal trauma or through surgical resections of tumors and infections. Defects are considered nonunions when normal biological processes of healing have failed and require further intervention. There are many definitions of nonunions in the literature, many of which have a temporal component. For the purposes of clinical investigation, the US Food and Drug Administration defines nonunions as defects that are nine months old and have shown no radiographic progression of healing for three months (Haverstock and Mandracchia 1998). This definition is often disregarded because of the inherent limitation of a temporal definition; the limitation being that variations in defect size impact healing time.

Nonunions can also be classified into one of three categories, each requiring a different approach to treatment. The three types are (1) hypertrophic, (2) oligotrophic, and (3) atrophic (Biasibetti et al. 2005). Each specific type underscores the mechanical and biological requirements for healing that have not been met.

(1) **Hypertrophic nonunions** are fractures that have begun the healing process but lack the appropriate mechanical stability to fully heal. A hypertrophic nonunion can be seen clinically with abundant callus formation that possesses adequate blood supply. In this situation the fracture has adequate biology to heal but lacks adequate mechanical
stability. This type of nonunion frequently heals with the addition of mechanical stability, without any biological stimulus.

(2) **Oligotrophic nonunions** are a class of fractures that are viable, possessing an adequate blood supply, but lack any callus formation. Treatment of these fractures usually includes a combination of mechanical stability in addition to a bone graft to improve the local biology.

(3) **Atrophic nonunions** are the most severe cases of nonunions and remain the most difficult to treat. Atrophic nonunions lack appropriate biology and present with visible bone resorption at the nonunion site on radiographs. Treatment requires significant intervention to increase the biological activity at the defect site. Current treatments are discussed below.

A significant aspect of fracture healing alluded to above is the presence of an adequate vasculature. Bone is a highly vascularized organ and issues that arise from compromised blood flow following the creation of a defect are well recognized (Kanczler and Oreffo 2008; Gerber and Ferrara 2000). However, the recognition of the importance of angiogenesis (generation of new blood vessels) in bone healing is a recent focus in the field. More recently, investigators have suggested that any future strategy for improving fracture healing should contain a component that increases vascularity (Einhorn 1995; Dickson, Katzman, and Paiement 1995; Giannoudis, Einhorn, and Marsh 2007; Glowacki 1998; Keramaris et al. 2008; Reed et al. 2003). Currently, clinicians are limited in their ability to affect fracture vascularity and prevent nonunions.
1.2 Bone Biology

Bone development is a coordinated sequence of events involving multiple tissue types and a complex array of paracrine factors (Colnot et al. 2005). The course of bone development begins at embryogenesis, continues after birth in the growth phase, and culminates in the continuous cycle of remodeling that occurs on a daily basis (Olsen, Reginato, and Wang 2000). It is from the embryonic bone formation pathway that fracture healing takes its cues.

Bone has several important cell types that regularly appear throughout its life cycle. The mesenchymal stem cell (MSC) is a precursor cell that will differentiate into bone and cartilage cells that build bone. Bone cells can be divided into three different subtypes based on function: osteoblasts (responsible for depositing bone matrix), osteoclasts (responsible for resorption of deposited bone), and the mature osteocyte. Osteocytes form when an osteoblast ceases forming new bone. Subsequently, osteocytes become surrounded by bone matrix and become responsible for maintaining aspects of homeostasis. Cartilage cells, or chondrocytes, form the cartilaginous structures in an adult. Articular cartilage surrounding bones in joints are examples of this more permanent type of cartilage structure. In both bone development and healing, chondrocytes often form the temporary structures that are replaced with bone. The temporary cartilage structure in bone lengthening is the growth plate. In fracture repair, that structure is the callus.
1.2.1 Bone Development

The origin of bone lies in cells from three distinct areas of the developing embryo: cranial neural crest cells that form the craniofacial elements (Bronner-Fraser 1994), sclerotomes that develop into the axial skeleton (Tam and Trainor 1994), and lateral plate mesoderm that create the limb buds (Cohn and Tickle 1996). Cells from these areas differentiate into MSCs that aggregate and condense in a fashion that patterns the future bone (Hall and Miyake 1992). At this juncture, condensed MSCs differentiate into chondrocytes or osteoblasts. Two types of bone formation exist dictating the initial differentiation of an MSC: intramembranous ossification (IO) and endochondral ossification (EO) (Marks Jr. and Odgren 2002).

1.2.2 Intramembranous Ossification

Intramembranous ossification is bone formation without a cartilaginous intermediate. This method of bone development is responsible for the formation of flat bones like that of the cranium and face. As mentioned previously, during development MSCs aggregate in clusters in areas of future bone formation. IO begins with MSC condensation, the invasion of capillaries into the mesenchymal zone (MSC aggregates), and the differentiation of MSCs into osteoblasts. These osteoblasts begin depositing bone matrix by forming individual spicules that grow and eventually connect to each other. When the bone spicules
connect, a disorganized network of trabeculae is created that is characteristic of woven bone. Due to the disorganized nature of woven bone, it is mechanically weak, and will eventually be replaced by a more organized network of lamellar bone.

1.2.3 Endochondral Ossification

EO is the method of bone development that occurs in the majority of bones in the human body. It differs from IO in that it has a cartilaginous intermediate. In EO, condensed MSCs differentiate into an avascular cartilage model of the future bone. At the center of this cartilage a number of chondrocytes become hypertrophic and cease to proliferate. Adjacent to these hypertrophic chondrocytes is another subset of cells differentiating into osteoblasts, forming a structure termed the bone collar. The hypertrophic chondrocytes are involved in initiating the invasion of capillary networks and osteoblasts while becoming apoptotic themselves. Apoptosis ensures space for cellular invasion to form the bone. This begins a cycle of chondrocyte proliferation, chondrocyte hypertrophy, invasion, apoptosis, and bone formation. The most active areas of EO are growth plates that continuously deposit the bone thereby lengthening the bone. The bone collar eventually becomes the cortices of the resultant bone.
1.2.4 Angiogenesis and Vasculogenesis

A healthy vascular network in bones is essential for homeostasis and restoration of bone, without which the bone would degenerate and die (Schmid et al. 1997). The importance of circulation was documented as early as the 1770s, however since then osteogenesis research has mainly focused on the role of the osteoblast (Kanczler and Oreffo 2008). Vascularity was reintroduced as a possible research target in 1963 when Trueta et al. published their work on what they called the vascular stimulating factor; a paracrine element released at the fracture site in defect models (Trueta 1963). The importance of the angiogenic and osteogenic relationship has been strengthened in recent years with research providing in vitro and in vivo evidence to this effect. Some examples of this evidence include endothelial cell-osteoblast interactions (Meury, Verrier, and Alini 2006), angiogenic factor-osteoblast interactions (Deckers et al. 2002), and data showing that compromised vascular networks lead to disease states like osteonecrosis (Childs 2005), osteomyelitis (Lazzarini, De Lalla, and Mader 2002), and osteoporosis (Burkhardt et al. 1987).

The mechanisms of blood vessel formation fall within two categories: generation and growth. Generation is the de novo development of vessels when precursor endothelial cells called angioblasts aggregate and form nascent networks in a process termed vasculogenesis. These networks are developed continuously through sprouting, bridging, and growth from existing vessels in angiogenesis. The highlighted difference between angiogenesis and vasculogenesis is the capacity to induce vessel formation with or without the presence of existing blood vessels respectively (Jain 2003).
Angiogenesis is a multistep process that involves vasodilation of the existing vessel, degradation of the basement membrane, liberation of the endothelial cells, and the migration/division of those cells to elongate the vessel. Angiogenesis is highly regulated by the balance of pro- and anti-angiogenic growth factors. In contrast, vasculogenesis begins with the differentiation of stem cells. *In utero*, those stem cells begin with hemangioblasts differentiating into hematopoietic stem cells (blood cell precursors) and angioblasts (the most primitive endothelial progenitor). Angioblasts aggregate in plexuses that differentiate into increasingly more mature vessel networks. Growth factors involved in vasculogenesis overlap with those involved in angiogenesis (Carmeliet 2000).

As mentioned previously, vascular invasion during bone growth occurs in IO and EO at different time points. In IO, capillaries begin invading the mesenchymal zone followed by the formation of calcified bone (Marks Jr. and Odgren 2002). In EO, as the chondrocytes become hypertrophic at the ossification center, they begin to release angiogenic factors like vascular endothelial growth factor (VEGF) (Gerber et al. 1999) and fibroblast growth factor (FGF) (Baron et al. 1994) that promote the invasion of vessels into the spaces where other hypertrophic chondrocytes have undergone apoptosis. This process is facilitated by the release of matrix metalloproteinases 2, 9, and 13 by hypertrophic chondrocytes and migrating cells from the nascent marrow (Hall, Westwood, and Wadsworth 2006).
1.3 Bone Healing

Defect healing in bone is a remarkable process because it is one of the few tissues in the body that heals without scarring. The physiological result of this ensures that the bone regains mechanical integrity to prevent re-injury. Consequently, the defect site becomes stronger when compared to its pre-injury state. To achieve this level of regeneration, bone heals in a fashion that mimics development.

The local environment that surrounds the bony defect will determine which type of fracture healing occurs. Healing is characterized by the formation of an intermediate tissue, the callus, and in conventional situations is slowly replaced by lamellar bone. The most important environmental factors that affect healing include the degree of stability in combination with oxygen tension and local growth factors. Depending on the pairing of these factors, the sequence of events will either be skewed towards primary healing, secondary healing, a combination of primary and secondary healing, or a failure to heal (Nauth et al. 2010a).

1.3.1 Primary Healing

Primary healing occurs in defects with a high degree of stability and high oxygen tension. This type of healing is not always achievable as the rigid fixation it requires is not always possible, or advisable, in some fracture patterns (eg comminuted fractures or long bone
fractures of the femur/tibia). Primary bone healing proceeds through the process of intramembranous ossification, similar to that of development of many flat bones. It is characterized by the direct formation of bone without the intermediate callus formation. Primary healing can be broken up into two subcategories based on the nature of the fracture: gap healing and contact healing.

Gap healing in primary bone formation begins with the formation of woven bone (hard callus) that is in a transverse orientation compared to the original lamellar bone orientation. After several weeks, remodeling replaces the woven bone, reconstructing the necrotic ends of the bone and creating the longitudinal network of lamellar bone. Contact healing occurs without the intermediate formation of woven bone. This situation involves the resorption of bone across the fracture line by osteoclasts followed by the movement of osteoblasts to replace the bone (Lieberman and Friedlaender 2005).

1.3.2 Secondary Healing

Secondary healing occurs in fractures that have an intermediate degree of strain and stability coupled with a lowered oxygen tension. This scenario promotes chondrocyte differentiation and cartilage formation (soft callus) that effectively increases the stability of the defect. Secondary healing occurs in three phases: the inflammatory phase, the reparative phase, and the remodeling phase. These phases tend not to be distinct and appear
to overlap one another. It can take years to achieve complete remodeling (Lieberman and Friedlaender 2005).

1. **Inflammatory Phase:** Trauma that damages a bone will undoubtedly damage local vasculature and surrounding soft tissues. The effect of this damage begins a cascade of events that initiates the healing process, peaking at 48 hours post injury and disappearing by the end of the first week. Endothelial damage causes the formation of a hematoma that encompasses the defect, effectively inducing the first measures to stabilize the injury in addition to causing pain and swelling. The clotting cascade and cells within the hematoma produce growth factors and chemotactic signals that induce the migration of progenitor cells alongside the angiogenic invasion of new vasculature.

2. **Reparative Phase:** Prior to the end of the inflammatory phase, the reparative phase begins and lasts for several weeks. This period is characterized by the invasion of mesenchymal stem cells that differentiate into fibroblasts, chondroblasts, and osteoblasts. These cells may originate in the damaged tissue or have migrated to the site of injury from the surrounding tissue and circulation. Osteocytes at the fracture ends become necrotic and the process of callus formation often begins as a combination of intramembranous and endochondral ossification.

3. **Remodeling Phase:** The normal activity of osteoclasts, responsible for the everyday resorption of bone, is accelerated in the fracture scenario. It begins and ends with the replacement of the callus with lamellar bone. This gradual process can continue for years after injury, influenced by mechanical loads until optimal stability is achieved.
1.3.3 Critical Aspects

As discussed above, bone healing is a complex series of events involving many cells, secreted signals, and genetic changes. A macroscopic view of healing is required to predict how a therapy can be effective. Bone healing itself can be divided into five components and a deficit in any of these could lead to a failure of healing. The five components are (Nauth et al. 2010a):

1. **Osteogenesis**: For a bone to heal, the defect must contain a sufficient population of cells that can create new bone. This can include progenitor cells, osteoclasts, or osteoblasts that are either present at the fracture site, or migrate from adjacent bone marrow, periosteum, muscle, and circulation.

2. **Osteoinduction**: The genetic cascade of bone healing is mediated by the release of soluble proteins known as cytokines and growth factors. The presence of cytokines induces migration of osteogenic cells to the site of injury. Once cells reach the injury site, growth factors can induce division, differentiation into effector cells, or the release of additional growth factors. Osteoinduction is the process by which osteogenesis is stimulated.

3. **Osteoconduction**: The environment encompassing the fracture site has a large effect on the motility and differentiation of cells. Extracellular matrix is the scaffold that cells migrate along and forms the framework of bone formation.

4. **Mechanical Stability**: The bony ends of the fracture must remain stable in an anatomically correct position in order to allow complete healing. The degree of stability
can determine the type of healing or lack thereof. A high degree of stability also protects the area from re-injury.

5. **Vascularity:** There is an established connection between bone formation and blood vessel formation in both development (Gerber and Ferrara 2000) and healing (Glowacki 1998). Inadequate vascularity is often a problem in cases of nonunion (Lu et al. 2007).

These factors have been conceptualized in what is termed “The Diamond Concept.” (Giannoudis, Einhorn, and Marsh 2007) It is a visual representation of how all the factors are connected in a cyclical manner, where each factor hinges on another. With four factors comprising the sides of the diamond, the idea that each one hinges on vascularity reveals its importance. Contained within the diamond is the “host” or the specific patient related factors mentioned above (Figure 1-1) (Giannoudis, Einhorn, and Marsh 2007). An example of how this would apply would be an elderly patient who has an increased chance of nonunion because of the diminished capacity at every aspect.

These critical concepts relating to the healing process can be applied to current therapies used to treat orthopaedic issues as well as the development of new therapies. Bioengineers can look at this concept and deduce a therapy that enhances one aspect or targets all aspects.
Figure 1-1 The Diamond Concept

Graphic representation of the critical components of fracture healing. The four sides represent four critical components hinging on the fifth and most important component, vascularity. Contained within these critical components are specific host factors that will influence healing.

1.4 Current Treatments

Current clinical treatments are limited by a number of issues. Often treatments have complications that can be severe and the effectiveness of treatments are not ideal. The search for a novel treatment must address these limitations.

Treatment of bone defects – from simple fractures to complex comminuted fractures – begins with stabilization using either external or internal hardware. With a proper anatomical positioning and stability, a fixed fracture can begin the healing process. The evolution of fixation techniques has followed the advancement in the biological understanding of fracture repair. Large open surgeries to achieve absolute stability have been replaced by less invasive surgeries that utilize devices to achieve relative stability (Perren 2002). These devices and the techniques used to implant them sacrifice the absolute stability in favor of limiting disruption of the surrounding soft tissue and vascularity (Farouk et al. 1999). Evidence supporting the use of less disruptive techniques includes increased callus formation, increased union rates, decreased union times, fewer infections, and complications (Papakostidis et al. 2006; Schutz et al. 2001).

Occasionally, despite a surgeon’s best efforts, the defect fails to heal, resulting in nonunion. There are a number of treatments currently available to treat nonunions. The current gold standard of treatment is the autogenous iliac crest bone graft (AICBG). The procedure consists of harvesting cancellous bone from the iliac crest of the patient with the
nonunion. The graft is then applied to the injury site (Sen and Miclau 2007). AICBG treatment targets three of the critical aspects of bone healing. (1) The bone and aspirate that are taken from the iliac crest contain progenitor cells that are able to undergo osteogenesis. (2) The cancellous structure of the graft provides a natural scaffold that aids osteoconduction. (3) There is a limited quantity of osteoinductive proteins, such as bone morphogenic proteins (BMPs), that stimulate bone growth (Bauer and Muschler 2000).

The benefits of AICBG include histocompatibility with donor, no risk of disease transmission, and strong biological characteristics. The graft, however, fails to provide mechanical stability and does not address vascularity. AICBG is also limited by a number of complicating factors including: donor site morbidity, limited quantities of graft material, pain, infection, hematoma, and fracture. Therefore, while AICBG remains the gold standard of augmenting treatment, it fails to be the ideal treatment that orthopaedic clinicians need (Sen and Miclau 2007). Alternative graft sites and techniques are being investigated to reduce the complications and to increase graft material (McCall et al. 2010).

Another type of bone graft in use is allograft bone. This bone is usually harvested from deceased tissue donors and therefore can be used off the shelf. Because of sterilization protocols, the main use of these grafts is to promote osteoconduction and to increase mechanical stability. The risks of this particular graft include immune responses and an increased risk of disease transmission. Due to their limited effectiveness, allografts are usually confined to cases in which there is a large structural deficit and are often used in
conjunction with AICBG (De Long et al. 2007). Figure 1-2 illustrates the current treatment of problematic fractures.

One alternative to bone grafts is the local application of BMPs. There are two recombinant human forms available for use in North America and Europe: BMP-2 and BMP-7. Both have been investigated in a number of clinical settings including: open tibial fractures (Govender et al. 2002), segmental defects (Jones et al. 2006), and nonunions (Friedlaender et al. 2001). These studies have shown, at best, equal effectiveness when compared to AICBG without the complications associated with graft harvest. A major detractor from widespread adoption is the high cost of these proteins, leading some to question cost-effectiveness (Nauth et al. 2009). Additionally, reports of induced swelling at surgical sites can lead to significant complications (Perri et al. 2007).

There are many other therapies in pre-clinical and early clinical phases of trials, all of which have yet to show conclusive effectiveness in comparison to AICBG. Some of these include: alternative growth factors and systemic agents like parathyroid hormone and platelet derived growth factor (PDGF), bone graft substitutes such as synthetic matrices, mechanical stimulation with ultrasound and electrical stimulation, and a number of cell and gene based therapies that will be discussed below (Nauth et al. 2010a). All of these therapies will subsequently need to be compared to the current gold standard of AICBG.
Figure 1-2 Current Treatment

Radiograph of 18-year-old patient suffering from an open tibial fracture, initially treated with closed reduction and cast immobilization. Left: Nonunion. Right: Solid union formed 3.5 months after compression plating and iliac bone graft. Illustration of modern techniques used to treat nonunion that include mechanical stabilization and bone grafting.

1.5 Scaffolds

As mentioned previously, one of the critical aspects of bone healing is the presence of an osteoconductive scaffold, a material that facilitates the migration and differentiation of cells into nascent bone. Current therapies already utilize a number of scaffolds while further research is being conducted to engineer newer materials that will have superior osteoconductive effects. Currently used scaffolds include calcium phosphates, calcium sulfates, and demineralized bone matrix.

Calcium phosphate is a ceramic with a highly crystalline structure that is suited towards osteoconduction. Included in its advantages is that it is available in a variety of formulations including pellets and cement. Further advantages to calcium phosphate include the off-the-shelf use, the high mechanical strength, low morbidity, and low risk of disease transmission. Disadvantages include poor tensile strength and the low ability to affect the other critical aspects of bone healing. The best evidence for use as a bone graft substitute in the management of subarticular metaphyseal defects (Bajammal et al. 2008).
Calcium sulphates are an older class of scaffolds that are resorbed at a faster rate compared to calcium phosphates. The fast rate of resorption limits the osteoconductive capacity of this particular scaffold. The availability in combination with antibiotics makes it a useful scaffold in osteomyelitic defects (McKee et al. 2002).

The final scaffold used in current therapies is demineralized bone matrix (DBM). DBM is the extracellular matrix of allograft bone that is demineralized through acid extraction. It is highly osteoconductive but offers little mechanical strength. There is a theoretical potential for disease transmission due to the allograft nature of the product. It is recommended that this scaffold be used in combination with AICBG (De Long et al. 2007).

There are countless novel scaffolds in development and under study currently. They are either natural or synthetic polymers encompassing a number of different materials including but not limited to ceramics, metals, collagens, chitosan, and silk. There are also composite materials including two or more of these substances as well as materials embedded with growth factors and cells. The endless combination creates a large field of study to investigate the superiority of one over another (Hagen, Gorenoi, and Schonermark 2012).
1.6 Tissue Engineering

To meet the critical requirements of fracture healing, research has progressed along numerous different paths to achieve the same goal; to increase the rate of healing and to decrease the number of nonunion formations. Poly-therapy with more than one novel technique is a potential alternative to any single treatment. Tissue engineering is the investigation into applying multiple components to create new tissue for therapeutic purposes. Limitations of current therapies in clinical orthopaedic practice present opportunities for tissue engineering to improve care.

As previously stated, many of the current alternatives being utilized in clinical settings fail to achieve an efficacy that compares to AICBG. One possible reason for these results is that while enhancing one critical aspect of fracture healing may offer mild benefits, the failure to achieve enhancement of more than one aspect may limit potential (Bauer and Muschler 2000). Tissue engineering affords the clinician a potential therapy that can effectively target more than one component of healing.

Current in vivo studies are beginning to examine the potential application of these therapies at a smaller scale. They focus on delivering cells and growth factors to a fracture site to augment healing rate. Two common methods being investigated are gene therapies and cell-based therapies (CBT). These two strategies overlap in orthopaedic models based on the need for an effective treatment.
One modality of gene therapy seeks to utilize cellular agents and enhance their capacity to induce bone formation when delivered to a defect area. Cell-based gene therapy involves the transfer of therapeutic genes into cells with the goal of altering their physiological characteristics. Cells can be subsequently implanted directly to the defect site where the gene product can affect change. While research is ongoing, there have been limited clinical applications.

Preclinical models investigating the efficacy of this modality of treatment are currently underway. Animal studies utilizing various models of *ex vivo* gene therapy using BMP-4 (Rose 2005), BMP-2 (Virk 2011), VEGF (Li 2009), and FGF (Guo 2006) genes have all shown bone formation and healing at the defect site. Initial clinical application of cell-based gene therapy in orthopaedics targeted arthritis, which demonstrated the safety and validity of gene therapy within the self-contained joint capsule (Bandara 1992). These studies remain in preclinical stages because this treatment modality is still considered experimental. Cell based gene therapy is also being investigated in other clinical fields.

Despite potential, cell-based gene therapy has stalled in translation to clinical practice. Numerous social and scientific factors may contribute to barriers preventing the approval of such studies. While complex factors keep gene therapy at preclinical stages, it remains a viable avenue for further research. The future of cell-based gene therapy in orthopaedics requires translational work to advance (Evans, Ghivizzani, and Robbins 2012; Nauth et al. 2010b).
CBT is the application of various bioactive cells to stimulate healing. In orthopaedics, this is achieved by augmenting various components of fracture repair. CBT is a promising adjunct to existing therapies because there is little manipulation of the cells that are taken from patients. Cells are isolated and expanded *ex vivo* from the patient, bypassing immunological complications. There are a number of cell types that can be utilized to augment the necessary components of fracture healing, usually by osteogenesis or angiogenesis. This rationale has focused the majority of research on two types of cells; the MSC for its ability to differentiate into osteoprogenitors and the endothelial progenitor cell (EPC) for its ability to induce neovascularization. Figure 1-3 depicts the application of cells for fracture repair. Research also exists on other less commonly used cells, one example being osteoblasts.
Figure 1-3 Cell Based Therapy

Illustration of the concept of CBT. A heterogeneous population of cells are extracted from a patient, expanded into specific cell types through differential culturing, seeded onto a scaffold, and implanted into a defect nonunion.

1.7 Endothelial Progenitor Cells

EPCs are a population of progenitor cells of haematopoietic origin that are known to influence blood vessel formation. It was previously thought that angiogenesis was the only method of vessel formation outside the womb. The discovery of EPCs altered the physiological dogma of postnatal vessel formation, displaying the capacity for adult vasculogenesis (Asahara 1997; Kearne 1999). *In vitro* and *in vivo* evidence of angiogenic and osteogenic potential (Kaufman 1997; Lewinson 2007; Mifune 2008) highlights EPCs as a promising cellular population in orthopaedic medicine. Additionally, adequate vascularization is a prerequisite for bone formation and inadequate blood flow at a fracture site contributes to nonunion. The utilization of EPCs in CBT has begun in clinical trials for a number of other vascular diseases and insults including ischemic limb disease, myocardial infarction, angina, and pulmonary hypertension (Wijns 2005; Losordo 2007; Wang 2007a; Kawamoto 2009; Burt 2010).

EPCs reside mainly in bone marrow, with small numbers found in tissues such as skeletal muscle and vascular parenchyma (Rafii 2003). Cytokine release during tissue injury and ischemia mobilizes EPCs from their niche, creating a movement of EPCs towards active circulation (Rabbany 2003; Urbich 2004). EPCs in circulation home to sites of vascular injury via chemotaxis towards angiogenic signals (Kluge 2000). There exists many studies in the literature describing isolation methods and this has resulted in subclassifications of EPCs by various population markers and functional potentials. EPCs have been shown to promote vascularization by direct integration into endothelium or through the release of
angiogenic paracrine factors like VEGF, stromal cell-derived factor 1-alpha (SDF-1α), hepatocyte growth factor (HGF), angiopoietin 1 (Ang-1), and insulin-like growth factor 1 (IGF-1) (Asahara 1997; Matsumoto 2006; Miyamoto 2007).

EPC-enriched populations have demonstrated a role in vasculogenesis during normal fracture healing. One report described an increase in circulating CD34+ cells and CD133+ cells by three days after a closed diaphyseal tibial fracture in humans (Tondreau 2005). This is similar to the fracture response observed in rat and mouse models (Matsumoto 2006). Studies using intravenously-delivered GFP+ EPCs show that EPCs migrate to fracture sites and contribute both directly and indirectly to vasculogenesis in response to fracture signals (Miyamoto 2007; Eghbali-Fatourechi 2005).

Evidence for EPCs role in supporting bone regeneration has been established, although the mechanisms have yet to be fully elucidated. The close connection between angiogenesis and osteogenesis suggests a possible role of EPCs in directly enhancing osteogenesis, separate from the healing benefit gained from increases in vascularity attributed to therapy. Pro-angiogenic factors expressed by EPCs affect osteoblast differentiation and survival (Dillon 2007; Matsumoto et al. 2008; Wang 2007b) and bi-directional communication between EPCs and osteogenic cells create environments permissive for bone formation (Street 2009; Jeong 2010). Evidence also suggests that EPC-enriched populations have direct osteogenic potential in vitro. CD34+ cells from human bone marrow have been induced to transdifferentiate into osteoblasts and G-CSF-mobilized
CD133+ EPCs isolated from circulation are capable of becoming osteogenic in culture (Kaufman 1997; Stahl et al. 2005). Additionally, a circulating population of cells has been shown to express osteocalcin. Initial studies in animals have shown that human-derived CD34+ cells form both endothelium and osteoblasts in a rat fracture model (Matsumoto et al. 2008; Grellier, Bordenave, and Amedee 2009). The mounting evidence that demonstrates a connection between EPCs, vascularity, and osteogenesis may yield a population that can exceed the potential for healing in vascular disorders (Figure 1-4).

Building on evidence of an osteogenic potential and a physiological role in fracture healing, EPC-based therapy has been experimentally shown to enhance healing past physiological limits. Atesok et al. (2010) demonstrated that bone-marrow derived EPCs on a gelfoam scaffold were able to completely heal critical-sized defects in a rat femur as compared to a scaffold-only control (Atesok et al. 2010). The EPC-treated group showed a complete osseus union at the defect site. Biomechanical strength was similar to that of a contralateral control (Li 2011). Similar results have been reported using G-CSF-mobilized EPCs isolated from peripheral blood in a model showing dose-dependent healing (Mifune 2008). EPCs have also been successfully applied to fracture nonunions in larger animal models, including sheep (Rozen et al. 2009). In the first reported clinical orthopaedic use of EPCs, autologous G-CSF-mobilized CD34+ cells in combination with an autologous bone graft successfully treated a tibial nonunion, with no adverse symptoms or side effects (Kuroda 2011). The first clinical trial using EPCs for treatment of fracture nonunion is ongoing.
Figure 1-4 EPC Recruitment to Bone

Schematic detailing the recruitment of CD34+ cells/EPCs during fracture healing to induce vasculogenesis/angiogenesis. CD34+ cells are stimulated to enter circulation from surrounding areas of bone callus. These cells form new blood vessels within the callus as well as releasing VEGF to promote vessel formation. CD34+ cells are also thought to enter the callus vasculature and undergo osteogenesis to promote bone formation.

Limited clinical adoption in spite of promising pre-clinical data is due to certain gaps in the literature. The first gap is the lack of a singular effector population. Many definitions have been given to this cell type and no singular surface marker exists to identify this progenitor population during isolation procedures (Calzi 2010). Concurrently, although EPCs have demonstrated the ability to induce bone formation, precise mechanistic research is required to elucidate their role in the interaction between angiogenesis and osteogenesis. Finally, EPC dysfunction in vascular diseases (Krenning 2009; Grant 2010) and their contribution to tumor formation (Shaked 2008) may limit application of the cell type in certain patient populations and raise questions of safety.

1.8 Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs), also known as multipotent stromal cells, were identified in the 1960s by Friedenstein et al. (Friedenstein, Piatetzky-Shapiro, and Petrakova 1966) Initial observations of this population yielded a cell type with capabilities to differentiate along a mesenchymal lineage in vitro. Thus, the defining progenitor characteristic of an MSC has been its ability to differentiate into bone, fat, and cartilage. Scientists quickly recognized the capacity to differentiate into bone and cartilage could be utilized as a therapy for a variety of orthopaedic disorders. In the decades following their discovery, MSCs have been extensively characterized to illuminate their potential.

MSCs are rare in the bone marrow, constituting less than one percent of the mononuclear cell population. However, MSCs can be easily expanded ex vivo up to ten passages before diminishing in proliferative capacity (Javazon, Beggs, and Flake 2004). With the lack of a
singular marker to identify functional MSCs grown *ex vivo*, the scientific community has sought to reach a consensus on the characteristics that define a true MSC. One group has established three criteria: (1) MSCs must be plastic-adherent when maintained in standard culture conditions; (2) must express CD105, CD73 and CD90, and lack CD45, CD34, CD14 or CD11b, CD79alpha or CD19 and HLA-DR surface molecules; (3) and MSCs must be able to differentiate to osteoblasts, adipocytes and chondroblasts under the appropriate conditions *in vitro* (Dominici et al. 2006).

Despite the stringent lineage related definition imposed on them, MSCs have demonstrated an enormous plastic potential that ranges phenotypes from every dermal layer *in vitro*. In addition to the three previously stated cell types, MSCs have been differentiated into myocytes, neurons, endothelial cells, and insulin producing cells. MSCs have also been linked to pericytes, the population of cells that support the vascular framework. It is currently theorized that a large cohort of pericytes are MSC in origin (Crisan et al. 2008). Furthermore, MSCs are highly active immunoregulators that suppress the immune response by a paracrine mechanism (Salem and Thiemermann 2010). Unfortunately, despite the large amount of *in vitro* evidence pertaining to the potential, no single *in vivo* mechanism of healing has been elucidated. Figure 1-5 demonstrates the current field of *in vitro* MSC research.

MSCs have largely been successful when applied to animal models of bone healing (Kon et al. 2000; Richards et al. 1999; Petite et al. 2000). Locally injected MSCs resulted in a significant increase in bone volume, through formation of new trabecular bone and osteoid in a rat femoral distraction model (Richards et al. 1999). When MSCs were delivered via
hydroxyapatiteceramic (HAC) scaffold to a tibial gap in sheep, results indicated greater bone formation in comparison to the HAC-only control two months after surgery (Kon et al. 2000). Other animal studies have demonstrated that MSCs are a promising population for the treatment of a variety of orthopaedic problems including segmental bone defects (Petite et al. 2000), major osteochondral defects of the knee joint (Wakitani et al. 2011), and tendon tears (Tuan, Boland, and Tuli 2003). Finally, MSCs have potential beyond their physiological healing capacity by providing a vehicle for targeted gene therapy in orthopaedics. In a mouse model, BMP2-transfected MSCs increased angiogenesis and induced significantly more bone growth compared to direct application of BMP2 (Moutsatsos et al. 2001). Despite the evidence supporting use of MSCs in a variety of pre-clinical models, MSCs have had limited engagement in clinical settings.
**Figure 1-5 MSC Summary**

Summary of the current status of MSC research. Summary includes potential sources and characterization of therapeutic populations as well as functional capabilities. Potential uses in addition to potential challenges are listed.

While no one clinical study provides compelling evidence regarding MSC application in orthopaedics (Novicoff et al. 2008), various cases have demonstrated that MSCs can be an effective treatment for nonunions and large bone defects (Quarto et al. 2001; Wakitani et al. 2011; Wakitani et al. 2004; Gan et al. 2008; Davatchi et al. 2011). Quarto et al. (2001) performed the first reported MSC transplantation in which three patients with large segmental bone defects (4.0-7.0 cm segment of the right tibia, ulna and humerus) were treated with bone marrow-derived autologous MSCs. The cells were delivered to the defect site via macroporous hydroxyapatite scaffolds, integrating adequately and allowing the patients to regain limb function. Significant callus formation was seen radiographically after two months, a result the authors believe would take 12 -18 months with a traditional bone graft, providing encouraging results to continue clinical application (Quarto et al. 2001).

Other clinical studies have combined MSCs with various pro-osteogenic scaffolds and bioactive factors to improve bone healing in various orthopaedic scenarios (Gan et al. 2008; Kitoh et al. 2004; Bajada et al. 2007). The combination of autologous MSCs and platelet-rich plasma was shown to accelerate the speed of bone regeneration applied directly during distraction osteogenesis in three patients (Kitoh et al. 2004). In another case, a nine-year tibial nonunion resistant to six previous surgical procedures was treated with a combination of MSCs and calcium sulphate (CaSO₄) pellets. Two months after the experimental treatment, the nonunion was radiologically healed (Bajada et al. 2007). Combination MSC therapy has also been successfully applied in therapeutic bone fusion. In a group of 41 patients, Gan et al. (2008) combined MSCs with β-tricalcium phosphate in an attempt to enhance vertebral union. Ninety-five percent of cases in this small cohort
exhibited fusion (Gan et al. 2008). Although these clinical cases offer encouraging results for continued MSC therapy in various areas of orthopaedics, the absence of a large, well-designed, randomized clinical trial is an ongoing issue of contention amongst clinicians and scientists in the field.

Although MSCs have been successfully applied in clinical cases, the exact mechanisms and the optimal conditions for fracture healing are still mostly unknown. One study highlighting this uncertainty found that the maturity of implanted cells determined the source of new bone as well as the method of ossification. Subcutaneous implantation of MSCs in mice induced endochondral ossification of host origin cells compared to osteoblasts that underwent intramembraneous ossification of the donor cells (Tortelli et al. 2010). It is important for future basic science research to continue to improve upon the understanding of specific mechanisms and optimal conditions for MSCs to promote fracture healing. Future research should also focus on providing clinicians with evidence obtained in randomized controlled trials for all iterations of MSC therapy.

1.9 EPC vs MSC Therapy

Described above are a number of studies that have sought to determine the effectiveness of implanted progenitor cell populations in a critical-sized defect model. The purpose of these was limited to specific populations that had biological value in the healing process. MSCs have been used for a number of years in many models with a large number of in vitro studies to suggest possible mechanisms. EPCs are relatively novel to the field of
orthopaedics as a potential group of bioactive cells. Nevertheless, previous studies failed to address the issue of which population had more potential as a clinical therapy.

After demonstrating the effectiveness of EPCs in a defect model (Atesok et al. 2010), our group provided evidence for the superiority of EPCs over MSCs in the same model (Nauth 2012). Results showed radiographic healing at 10 weeks post-implantation (Figure 1-6), increased bone formation with microCT, and increased blood flow two weeks post implantation, all in favor of EPC therapy. The absence of healing seen with the MSC group highlights the unknown \textit{in vivo} effect that a progenitor cell can exert. A recent review of 18 studies was done comparing the use of EPCs/endothelial cells and MSCs in orthopaedic models. The authors found that co-implantation demonstrated increased bone formation compared to other implant scenarios in the majority of studies (Keramaris et al. 2012).

The data presented above demonstrates the clinical value of one progenitor population over another. What it fails to do is present evidence as to the reason for this discrepancy. A number of studies previously mentioned reference specific populations of MSCs that show bone regeneration in animal models. Being able to utilize a model where mechanisms can be compared will further elucidate the optimal population. The study shows that a lack of understanding with regards to \textit{in vivo} fate only leads to theories as to therapeutic effect.
Figure 1-6 EPC vs MSC Radiographs

AP Radiographs comparing progressive healing of a bone defect in a rat femur between MSC treated defects and EPC treated defects. The same rat is shown in each series. Callus formation is seen only beginning in the 4 week EPC group with healing achieved by 10 weeks. No healing is seen in the MSC group.

2 Aims

2.1 Rationale

Current standards of care fail to meet the clinical need to augment bone healing. The gap is clear when examining the costs of injury, including economic costs and measures of patient well-being. Previous research into CBT has shown the potential of MSCs and EPCs to induce bone healing. In addition, recent research has shown the potential superiority of EPCs over MSCs when applied for bone regeneration in an animal model. However, several critical breaches exist in our current knowledge of EPC and MSC therapy for fracture healing. First, the aforementioned investigation comparing EPCs and MSCs failed to fully define the two cell populations on the basis of accepted cell surface markers. Second, the in vitro differentiation potential and angiogenic potential of the two cell types has not been compared. Third, the fate of these transplanted cells has not been completely investigated and compared in animal models. Finally, the in vivo angiogenic response to EPC and MSC therapy has not been fully compared.

The present study sought to address these issues with a combination of in vitro and in vivo investigation. The goal was to provide a comprehensive comparison of the two cell types with regard to their in vitro and in vivo potentials for osteogenesis and angiogenesis, with the ultimate objective being a better understanding of the best cell population for augmenting fracture healing in a clinically useful manner.
2.2 Hypothesis

The thesis seeks to answer a number of questions surrounding the use of EPCs and MSCs in therapy targeting bone healing. The central hypothesis of this study is that characterized populations of EPCs and MSCs can be examined within a fracture callus after the healing process has begun.

The investigation initially begins with the examination of phenotypic and functional characteristics of the isolated EPCs/MSCs. From this we can answer questions regarding the identity of implanted cells and be able to further link these populations to their therapeutic effect. Further examination into phenotype and EPC/MSC potential to alter lineage can show a certain amount of plasticity between cell types.

The second major question that is being asked pertains to the fate of implanted cells in a CSD model. Demonstrating a continued presence of cells can allow further insight into mechanisms of healing in addition to providing a new base for further investigation. Additionally, in conjunction with the fate of cells, an investigation into the consequences of cellular implantation on the vascular network within the defect is being done. This is reasoned by the angiogenic nature of the cell line in opposition to MSCs. Vascularity, being a critical component of bone healing, is an important variable to examine. The effect of either population will give insight into mechanisms of healing.
2.3 Objectives

1) Characterization of EPC and MSC cell populations on the basis of cell markers and functional assay.

In order to fully characterize the populations of EPCs and MSCs that have been used for CBT, quantitative flow cytometry was used to examine the percentage of cells in culture expressing cell surface markers typical of these populations. Functionally, EPCs were tested for their ability to bind lectin, take up acetylated LDL.

2) Comparison of in vitro differentiation potential and angiogenic potential

EPCs and MSCs were compared in vitro under specific differentiation conditions including osteogenic, chondrogenic, and adipogenic. In addition their angiogenic capacity in vitro was compared by assessing tube formation on Matrigel™. Finally, MSCs subjected to endothelial growth media (EPC conditions) were assessed for tube formation on Matrigel™ to evaluate their capacity for transdifferentiation into an endothelial/angiogenic population.

3) Examination of cell fate in vivo

In order to assess the fate of EPCs and MSCs in vivo, green fluorescent protein (GFP) labelled cells were implanted into a critical sized bone defect model in a syngenic rat. Fate of the cells were assessed by immunohistochemistry (IHC) for the GFP protein at 2 and 4 weeks post-surgery in the developing fracture callus.
4) *In vivo* angiogenesis

In order to compare the *in vivo* potential of EPCs and MSCs when used for CBT, fluorescent microangiography (FMA) was used to examine the effect of each cell type on the early formation of vasculature in a critical sized bone defect model in a syngenic rat.
3 Methods

3.1 Cell Isolation and Culture

EPCs were isolated and cultured from syngeneic Fischer 344 rats using previously described methods (Atesok et al. 2010). Briefly, adult rats (250-300 grams) were anesthetized in a 5% isofluorane chamber and sacrificed by cervical dislocation. After sterile isolation of the femurs and tibiae, the bone marrow was flushed into a 50ml falcon tube using a syringe. The aspirate was subsequently centrifuged, re-suspended in warm media, placed on a ficoll-paque gradient, and centrifuged at 400xg for 30 minutes. The “buffy coat” layer consisting of mononuclear cells was carefully aspirated and re-suspended in Endothelial Basal Media (EBM2, Clonetics) supplemented with an EGM2-MV SingleQuots bullet kit [containing VEGF, R3-insulin-like growth factor-1 (R3-IGF-1), human epidermal growth factor-B (hEGF-B), hydrocortisone, gentamycin -amphotericin (GA-1000), 5% fetal bovine serum (FBS), human fibroblast growth factor (hFGF-B), and ascorbic acid]. The cells were then plated onto a T75 flask coated with human fibronectin and placed in an incubator at 37 °C and 5% CO₂. After 4 days of culture, non-adherent cells were removed by a PBS wash and fresh media was applied. Media was replaced every 2-3 days onward. Cells were used for all assays 6-11 days after isolation.

MSCs were cultured in a similar manner. Bone marrow was collected and processed in an identical fashion. The mononuclear cells aspirated from the “buffy coat” were re-suspended in alpha modified minimal essential media (α-MEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Cells were then cultured on an uncoated T75
flask. Non-adherent cells were washed after 4 days and media was changed every 2-3 days onward. Cells were passaged with 0.1% trypsin-EDTA after 7 days in culture and further passaged at 90% confluence. Cells from passages 3-7 were utilized for all experiments.

### 3.2 Flow Cytometry

Cells were isolated, and flow cytometric analysis was performed on EPCs 7-11 days after culture and MSCs at P3-P5. All flow cytometry was done using a MACSQuant (Miltenyi Biotec) to obtain the percentage of single cells that express markers typical of the population. EPCs were incubated with the following conjugated antibodies for 20 minutes at 4°C: PE anti-human CD133 (Miltenyi Biotec), V450 anti-mouse VEGFR2 (Flk-1) (BD Biosciences) and AlexaFluor 647 anti-mouse CD34 (Biolegend). MSCs were incubated with the following conjugated antibodies for 20 minutes: Brilliant Violet 421 anti-rat CD90, PE anti-human CD105, and APC anti-human CD73 (all from Biolegend). For both populations, one sample was incubated without antibody as a negative control and one sample was incubated with a combination of three antibodies to identify the triple positive population. Gating strategies were based on a negative control (unstained cells) as well as a FMO (fluorescence minus one) controls.
3.3 EPC AcLDL Uptake and Lectin Binding

EPCs were seeded on a 2 well chamber slide coated with human fibronectin at a density of 4000 cells per square centimeter. Following an incubation at 37ºC for 4 hours, 10 µg/ml AcLDL was added to each well and incubated for another 4 hours. Cells were then washed and fixed in 2% PFA for 10 minutes. Excess PFA was washed with PBS and 20 µg/ml lectin was added to each well and left at room temperature overnight. Lectin was removed with a PBS wash and the slides were mounted in medium containing a DAPI counterstain.

3.4 Mesenchymal Differentiation

In order to compare mesenchymal differentiation capacity, both EPCs and MSCs were subject to identical differentiation conditions. Initial differentiation of MSCs and EPCs were done with a Rat Mesenchymal Stem Functional Identification Kit (R&D Systems). For adipogenic differentiation, cells were seeded on a sterile coverslip and incubated in adipogenic media (containing hydrocortisone, isobutylmethylxanthine, and indomethacin) for two weeks. Coverslips were removed from the media, fixed in 4% paraformaldehyde, and stained with an anti-FABP4 primary antibody (an antibody specific to a carrier protein for fatty acids found in adipocytes) and a fluorescent secondary.

For chondrogenic differentiation, cells were initially suspended in chondrogenic medium (containing dexamethasone, ascorbate-phosphate, proline, pyruvate, recombinant TGF-
beta3, and recombinant BMP2). The resulting pellet was incubated for three weeks, after which was immersed in O.C.T. compound (Tissue-Tek), frozen with liquid nitrogen, and sectioned using a cryotome (Leica). Sections were subsequently stained with an anti-aggrecan antibody (an antibody specific for a protein expressed in chondrocytes) and a fluorescent secondary. Control samples were taken from each group including undifferentiated EPCs and MSCs as well as a secondary antibody only control.

For osteogenic differentiation, cells were cultured on a sterile coverslip and incubated in osteogenic media (containing dexamethasone, ascorbate-phosphate, beta-glycerophosphate, and recombinant BMP2) for three weeks. Coverslips were subsequently removed and fixed in 4% paraformaldehyde. Slides were then stained with an anti-osteocalcin antibody (an antibody specific to protein expressed in bone) and a fluorescent secondary.

Further osteogenic differentiation of EPCs and MSCs was carried out with media described previously. Fresh alpha-MEM was supplemented with 10% FBS, 2% penicillin-streptomycin, 200 µM L-ascorbic acid, 10mM beth-glycerophosphate, and 10⁻⁷ M dexamethasone. EPCs and MSCs were cultured for three weeks. Mineralization was confirmed with Von Kossa staining. Briefly, cells were incubated with 5% silver nitrate for 30 minutes at room temperature. Following a wash with distilled water, cells were then incubated with 5% sodium carbonate formaldehyde for 5 minutes. Cells were then washed with tap water and counterstained with methyl green pyronin for 20 minutes, washed, and dehydrated with 95% ethanol.
3.5 Endothelial Differentiation and Tube Formation

To induce endothelial differentiation of MSCs, cells taken from P3-5 were cultured in EGM2-MV for 3 weeks. Cells were passaged at 90% confluency. Cells cultured after 3 weeks were designated eMSCs.

Tube formation assays were carried out on MSCs, EPCs, and eMSCs. Briefly, 3x10^4 cells were seeded into each well of a 24-well plate coated with Matrigel. After 16 hours of incubation at 37°C and 5% CO₂, the wells were imaged on an inverted light microscope. A blinded grader quantified tube formation by overlaying lines connecting two cell bodies on image processing software (ImageJ) and statistical analysis was performed using a one-way ANOVA followed by Tukey’s post-hoc analysis. A confidence interval of 95% was set and a value of p<0.05 was considered statistically significant (GraphPad Prism).

3.6 In Vivo Experimental Design

The experimental design is briefly outlined in Figure 1-3. Three experimental groups were created. The control group consists of an implanted gelfoam scaffold soaked in saline. The EPC group contains 1 x10^6 EPCs on the gelfoam scaffold implanted directly into the defect. The MSC group contains 1 x 10^6 MSCs on a gelfoam scaffold implanted into the defect. The number of animals in each group is depicted in Table 3-1.
Each group was examined for either histology/IHC or with FMA. The histology/IHC groups were implanted with transgenic green fluorescent cells. Animals in each experimental group were either sacrificed at two weeks post-surgery or four weeks post-surgery. Half the animals sacrificed at two weeks were examined for histology/IHC. The other half of animals sacrificed at two weeks underwent FMA. All animals at four weeks were examined for histology/IHC.

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3.7 Cell Isolation and Seeding

Cells were isolated as previously stated. EPCs and MSCs were isolated from bone marrow of Fischer 344 rats. For histology/IHC, EPCs and MSCs were isolated from F344-Tg[UBC-EGFP] rats (RRRC) and implanted into wild type F344 rats (Charles River). EGFP-expressing rats are a commercially available strain of transgenic rats with a stable expression of green fluorescent protein on an ubiquitin promoter. Wild-type EPCs/MSCs were used for FMA investigation. EPCs were taken 6-11 days after isolation and MSCs were taken between P3-5.

On the day of surgery, cells (EPCs or MSCs) were detached from their flask with trypsin/EDTA, washed, and centrifuged. Following cell counting, 1 x 10^6 Cells (EPCs or MSCs) were then resuspended in 0.15 mL PBS and placed on a sterile piece of gelfoam (5mm x 5mm x 5mm) in a 1.5mL centrifuge tube. Seeding occurred 1 hour (at room temperature) prior to surgical transplantation.

3.8 Animal Model

The surgical model used (rat critical sized femoral defect) has been previously described (Atesok et al. 2010). Briefly, F344 male rats were anesthetized in a 5% isoflorane chamber and maintained at 2% isoflorane via nose-cone. The right leg was surgically sterilized and a lateral incision was made to expose the entire femur. An oscillating saw was used to create...
a 5mm osteotomy in the middle 1/3rd of the femur. The two ends of the femur were fixed with a miniplate and 4 1.5mm cortical screws: 2 distal and 2 proximal to the gap. Following fixation, the gelfoam scaffold containing either saline (control), EPCs (wt or GFP-EPCs), or MSCs (wt or GFP-MSCs). All layers of soft tissue were sutured in addition to the skin. Immediately after anesthetic recovery, full weight bearing and cage activity was allowed. St. Michael’s Hospital Animal Care Committee approved all animal protocols.

3.9 Histology/Immunohistochemistry

At 2 and 4 weeks post surgery, animals were sacrificed by cervical dislocation following induction of general anesthesia with 5% isofluorane. The entire femur was carefully removed and placed in 10% buffered formalin for 24 hours at room temperature. The callus was then carefully cut out from the gap and subsequently embedded in a paraffin block. Representative 5um sections were cut from the block using a standard microtome.

IHC was performed to enhance visualization of GFP+ cells. Briefly, sections were deparaffinized in xylene and rehydrated in a series of graded ethanol solutions. Antigen retrieval was carried out in a 10mM sodium citrate solution for 20 minutes at 98°C. Following antigen retrieval, the sections were exposed to 3% hydrogen peroxide in methanol and dako serum free protein block for 30 minutes each. Next, sections were incubated with chicken IgY anti-GFP diluted 1:500 overnight at 4°C. The secondary
antibody, a biotinylated goat anti-chicken diluted 1:200, was incubated for 30 minutes at room temperature. Streptavidin-HRP was added for 30 minutes and DAB for 10 minutes. Sections were counterstained with hematoxylin, dehydrated, cleared, and mounted. Sections were examined using a standard upright microscope.

3.10 Fluorescent Microangiography

At 2 weeks post-surgery, animals were induced into general anesthesia with 5% isofluorane and maintained at 2%. An incision was made in the abdomen to reveal the abdominal aorta, which was subsequently catheterized with a 22g catheter. The animal was then sacrificed with a T61 intracardiac injection. Blood was flushed with 30 mL PBS containing heparin and replaced with 10 mL of FMA microbead solution [1% low melting agarose, 1:10 0.2 µm carboxylate-modified FluoSpheres (Life Technologies)]. Following injection, the leg was placed on ice for 30 minutes. The femur was then removed and placed in 10% buffered formalin for 72 hours. After fixation, the callus was carefully excised and sectioned at 200 µm on a vibrating microtome (Leica). Sections were mounted on slides with mounting media for fluorescent samples. Slides were imaged on a confocal microscope (Zeiss) to create z-stack images. Images were processed on Neurolucida software to create 3-D renderings.
4 Results

4.1 Flow Cytometry

Representative cultures of EPCs and MSCs underwent flow cytometric analysis for cell-surface markers typical for their respective cell types. Cultures were analyzed by gating for single-cell populations based on established techniques. The first step in gating is the exclusion of debris and aggregates, which are excluded based on size and complexity. Further gating is done in the second graph to more accurately exclude aggregates of cells. The population of cells from this gate is considered single-cell in nature and is the population of cells in culture that is examined for fluorescence. Thus, percentage of positive cells is calculated in relation to this gated group.

**EPCs:** Isolation of bone marrow and selective culturing for EPCs yields a population of single cells where 98.5% express CD34, 98.5% express CD133, and 98.4% express VEGFR2. In addition, a total of 96% of single cells express CD34, CD133, and VEGFR2 concurrently (Figure 4-1).

**MSCs:** MSCs in culture between passages 3 and 5 express typical markers CD73 at 99.1%, CD105 at 98.5%, and CD90 at 99.2%. Furthermore, 94.7% of single cells in the same culture co-express CD73, CD105, and CD90. (Figure 4-2)
Figure 4-1 EPC Flow Cytometry

Immunophenotyping of EPCs with flow cytometry with gates selecting for cells (A) and single cells (B). The following graphs only display cells selected as single cells. Here we identify the percentage of cells expressing CD34 (C), CD133 (D), and VEGFR2 (E). These percentages are 98.5%, 98.5%, and 98.4% respectively. To show cells that express the three markers concurrently, cells positive for both CD133 and VEGFR2 were gated on CD34 positivity. Approximately 96% of single cells express all three markers.
Figure 4-2 MSC Flow Cytometry

Immunophenotyping of MSCs with flow cytometry with gates selecting for cells (A) and single cells (B). The following graphs only display cells selected as single cells. Here we identify the percentage of cells expressing CD90 (C), CD105 (D), and CD73 (E). These percentages are 99.1%, 98.5%, and 99.2% respectively. To show cells that express the three markers concurrently, cells positive for both CD105 and CD73 were gated on CD90 positivity. Approximately 94.7% of single cells express all three markers.
4.2 Functional Assays

A number of functional properties of EPC and MSC populations were examined for further characterization. EPCs were examined for their ability to bind to lectin in addition to AcLDL uptake. Figure 4-3 demonstrates a representative sample of cells where the majority of cells bind to fluorescent lectin and uptake fluorescent AcLDL. EPCs have also demonstrated the ability to form tubes \textit{in vitro}, as exhibited by a Matrigel tube formation assay in Figure 4-4. After a 16-hour incubation of a cell suspension, imaging under an ordinary light microscope reveals the formation of elongated connecting tubes between cell bodies.
Figure 4-3 EPC AcLDL uptake/Lectin binding

AcLDL uptake / Lectin binding assay. A) DAPI nuclear counter-stain B) Lectin binding C) AcLDL uptake D) Merged image
Figure 4-4 EPC Tube Formation

EPC tube formation on Matrigel™ 20x magnification
MSC are functionally defined by their ability to differentiate into osteocytes, chondrocytes, and adipocytes. Figure 4-5 depicts the induced populations expressing characteristic markers. MSCs that did not undergo the differentiation treatment were designated as a negative control. The controls displayed no visible staining for the markers seen in the differentiated populations. Osteoblast differentiation was assessed by the expression of osteocalcin, chondrocyte differentiation was assessed by the expression of aggrecan, and adipocyte differentiation was assessed by the expression of FABP-4. Mineralization occurring due to the osteogenic differentiation of MSCs can be seen with a Von Kossa stain (Figure 4-5C).
**Figure 4-5 MSC Mesenchymal Differentiation Assay**

MSC osteogenic (A-C), chondrogenic (D-E), and adipogenic (F-G) differentiation. A, D, F - Undifferentiated MSC negative control (20x). Osteogenic differentiation is shown with a positive osteocalcin stain (B) (20x) in addition to nodule formation visualized with Von Kossa stain (C) (4x). Chondrogenic differentiation is seen with positive aggrecan staining (E) (20x) and adipogenic differentiation is seen with a positive FABP-4 stain (G) (20x).
4.3 Cross Differentiation

To examine potential of EPCs and MSCs and possible mechanisms in fracture repair, EPCs were induced to differentiate along the mesenchymal lineage and MSCs were induced along an endothelial lineage (eMSCs). Mesenchymal differentiation of EPCs was examined under identical conditions that MSC differentiation utilized. Concurrently, endothelial differentiation of MSCs was examined functionally by comparing tube formation capacity between eMSCs, undifferentiated MSCs, and EPCs.

EPCs undergoing induction into osteocytes, chondrocytes, and adipocytes were stained for the same characteristic markers to determine if differentiation took place (Figure 4-6). EPCs that did not receive treatment were used as a negative control. Undifferentiated EPCs demonstrated a low level expression of the osteogenic marker osteocalcin in addition to a significant expression of the adipocyte marker FABP-4. EPCs undergoing osteogenic induction display an increased expression of osteocalcin, with the resulting culture phenotypically resembling the MSC culture differentiated into osteoblasts. Additionally, osteogenic differentiated EPCs display mineralized nodule formation. EPCs do not appear to undergo chondrocyte differentiation, with no visible aggrecan staining in either the control or induced population. As previously stated, undifferentiated EPCs express the FABP-4 marker that is used to determine adipogenic differentiation. FABP-4 expression of adipogenically differentiated EPCs appear to remain constant with no visible phenotypic difference from the control group.
Figure 4-6 EPC Mesenchymal Differentiation Assay

EPC osteogenic (A-C), chondrogenic (D-E), and adipogenic (F-G) differentiation. A, D, F - Undifferentiated EPC negative control (20x). Osteogenic differentiation is shown with a positive osteocalcin stain (B) (20x) in addition to nodule formation visualized with Von Kossa stain (C) (4x). Lack of chondrogenic differentiation is seen with the absence of aggrecan staining (E) (20x). Positive FABP-4 staining is seen before differentiation (F) and after differentiation (G) (20x).
Endothelial differentiation of MSCs was evaluated utilizing the Matrigel tube formation assay (Figure 4-7). This evaluation was compared to both undifferentiated MSCs and EPCs. After 16 hours of incubation on the matrix, both EPCs and eMSCs formed large networks of tubes. MSCs formed few tubes. A blinded grader subsequently quantified tube formation. In an evaluation of a series of random 20x visual fields, it was seen that MSCs (n=6) formed significantly less tubes (p<0.01) compared to both EPCs (n=6) and eMSCs (n=6). EPCs and eMSCs were not significantly different from one another.
Figure 4-7 Endothelial Differentiation Assay

Tube formation assay comparing endothelial differentiated MSCs (n=6) (A), MSCs (n=6) (B), and EPCs (n=6) (C). Graph depicting mean number of tubes counted in random 20x visual fields. * denotes p-value <0.01 Error bars indicate SEM
4.4 Immunohistochemistry

*In vivo* fate of implanted cells was conducted in a CSD rat femur fracture model. MSCs and EPCs from transgenic GFP rats were seeded onto a gelfoam scaffold and implanted into a 5mm gap created in the femur of an anesthetized rat. After fixation of the femur and suturing of the incision, healing was allowed for two and four weeks after surgery. The soft callus was subsequently excised with surrounding tissue. Post-processing, samples underwent IHC to visualize GFP-positive cells in bright field microscopy.

Representative sections from defects treated with saline-soaked scaffolds are seen in Figure 4-8 and Figure 4-9. Excision of the callus includes all the surrounding soft tissue to protect the integrity of the callus. A macroscopic view of a sample shows callus within distinct areas of muscle tissue. A notable result seen in samples at both two weeks post-op and four weeks post-op is a lack of specific staining by the antibodies used for this IHC protocol. What is visualized is a low level of background and nonspecific staining that is easily differentiated from specific GFP immunostaining. At two weeks post-op, large remnants of hematoma can be seen in Figures 4-8A and 4-8B. Hematoma remnants are not seen at four weeks post-op or in any of the treated groups. Figure 4-8C demonstrates the formation of fibrous tissue around remaining gelfoam scaffold. Figure 4-8D further illustrates the scaffold that has failed to be resorbed.

Figure 4-9, representative sections from the control group retrieved at four weeks post-op, shows healing progressing in untreated animals. Figures 4-9A and 4-9B demonstrates large amounts of scaffolding. Figures 4-9C and 4-9D display in closer detail the structure of the nascent tissue.
Figure 4-8 IHC: 2 Week Control

Representative sections of control fracture callus at 2 weeks post surgery (n=3). Anti-GFP IHC counterstained with hematoxylin. (A-B) 4x magnification (C) 10x magnification (D) 20x magnification
Figure 4-9 IHC: 4 Week Control

Representative sections of control fracture callus at 4 weeks post surgery (n=2). Anti-GFP IHC counterstained with hematoxylin. (A-B) 10x magnification (C-D) 20x magnification
EPC treated callus seen two weeks after surgery is demonstrated in Figure 4-10. Dark brown spots created by the staining reaction for GFP protein show a large number of cells remaining. The addition of these cells creates a clear contrast to the control group. Figures 4-10A and 4-10B are macroscopic views of callus structure. Figure 4-10A has a division between degrading gelfoam with cells lining the majority of the porous spaces and fibrous tissue forming around this area. Figure 4-10E is a close up of this border, demonstrating that a number of cells have become a part of the nascent fibrous network. Figure 4-10B is a section of a callus that contains less fibrous tissue and more scaffolding that is heavily populated with implanted EPCs. Figure 4-10C gives a closer view of the edge of the callus. EPCs are visible within both the fibrocartilage at the periphery that surrounds the callus in addition to more central areas. Figure 4-10D shows EPCs residing within central areas of the callus where remnants of the gelfoam appear to be. A notable observation of this section is that EPCs appear to be encircling areas of stained tissue. EPCs also appear to be very heterogeneous in morphology and location within the callus, demonstrated by the elongated “fibroblastic” cell type in Figure 4-10E compared to the rounded shape in Figure 4-10F, which also contains a structure that is encircled by implanted EPCs.
Figure 4-10 IHC: 2 Week EPC

Representative sections of EPC treated fracture callus at 2 weeks post surgery (n=3). Anti-GFP IHC counterstained with hematoxylin. (A-B) 4x magnification (C-D) 10x magnification (E-F) 20x magnification
Figure 4-11 contains representative sections from the EPC treated group at the four-week post-op time point. These samples appear to have as many implanted cells upon visual comparison with samples from the two-week group. The transition to bone formation has significantly progressed in two weeks while few remnants of gelfoam can be seen. Figures 4-11A and 4-11B show a macroscopic view of the samples, indicating that after four weeks, EPCs have populated the majority of the callus. EPCs are beginning to form larger structures and are taking on more varied morphologies.

Figure 4-12 demonstrates that like EPCs, MSCs survive within the callus after implantation two weeks prior. Within the MSC implanted group exists a lot of variability of a number of different factors. Examination of callus development reveals a large difference in tissue formation with some samples revealing large amounts fibrocartilage (Figure 4-12A) and some samples with little fibrocartilage (Figure 4-12B). Additionally, engraftment into nascent tissue ranges from high degrees (Figures 4-12A and 4-12C) to low degrees (Figures 4-12B and 4-12D). Figure 4-12E and 4-12F details the varying morphologies of implanted MSCs, ranging from elongated “fibroblastic” morphology to a rounded morphology.
Figure 4-11 IHC: 4 Week EPC

Representative sections of EPC treated fracture callus at 4 weeks post surgery (n=2). Anti-GFP IHC counterstained with hematoxylin. (A-B) 4x magnification (C-D) 10x magnification (E-F) 20x magnification
Figure 4-12 IHC: 2 Week MSC

Representative sections of MSC treated fracture callus at 2 weeks post surgery (n=3). Anti-GFP IHC counterstained with hematoxylin. (A-B) 4x magnification (C-D) 10x magnification (E-F) 20x magnification
After four weeks post-op, MSCs continue to be visible within the callus (Figure 4-13). Engraftment of cells appears to be variable between samples within this group (Figures 4-13A-4-13D). Figure 4-13A is representative of a sample with a larger number of visible cells when compared to samples represented by Figure 4-13B. Figures 4-13C and 4-13D demonstrate the localized nature of MSC tissue engraftment, whereby cells cannot be found in all areas of the developing callus. Implanted MSCs at four weeks post-surgery appear to be clustered in small discrete areas of the callus. Figures 4-13E and 4-13F illustrate MSCs undertaking both “fibroblastic” and rounded morphology. Figure 4-13F illustrates an example of an apparent blood vessel surrounded by stained cells. This result is not common within all samples.
Figure 4-13 IHC: 4 Week MSC

Representative sections of MSC treated fracture callus at 4 weeks post surgery (n=3). Anti-GFP IHC counterstained with hematoxylin. (A-B) 4x magnification (C-D) 10x magnification (E) 20x magnification (F) 40x magnification. Arrows highlight apparent vessels.
4.5 Fluorescent Microangiography

To examine the angiogenic affect of EPC/MSC treatment in fracture repair, FMA was employed to create 3D models of vascular structure within the callus two weeks post-treatment. Qualitative vascular structure is compared between a saline-soaked scaffold control, EPC treatment and MSC treatment. Vessel formation was subsequently quantified with the help of computer software.

Figure 4-14 illustrates maximum intensity projection (MIP) images taken from representative samples within each group. Figure 4-14A is the vasculature of a representative callus implanted with saline-soaked scaffold. Figures 4-14B and 4-14C are representative calluses from EPC and MSC groups respectively.

Figure 4-15 illustrates 3-dimensional renderings from representative samples within each group. Figure 4-15A is the vasculature of a representative callus implanted with saline-soaked scaffold. Figures 4-15B and 4-15C are representative calluses from EPC and MSC groups respectively.
**Figure 4-14 FMA MIP**

FMA of control fracture callus (A) (n=3) EPC implanted callus (B) and MSC implanted callus (C).

**Figure 4-15 FMA 3-D**

FMA of control fracture callus (A) (n=3) EPC implanted callus (B) and MSC implanted callus (C).
5 Discussion

Investigation into cell-based therapies for fracture repair has yielded many promising results. MSCs, being precursors to bone, are one of the most studied cell types in various bone healing models. In recent years EPCs have become another popular cell type in the field of orthopaedics due to encouraging pre-clinical data. The goal of this investigation was to examine progenitor populations isolated from bone marrow, characterize these cells with respect to phenotype and function, and relate the potential of these cells with their capacity to heal bone.

Through this investigation, EPCs and MSCs were shown to be phenotypically homogeneous by three positive markers each. EPCs were functionally characterized by their ability to uptake AcLDL, bind lectin, and form tubes in vitro. MSCs were functionally characterized by their ability to differentiate into bone, fat, and cartilage. EPCs, investigated for their mesenchymal abilities, demonstrated both a baseline osteogenic marker in addition to a heightened expression after differentiation. Furthermore, MSCs demonstrated angiogenic ability only after culture in endothelial media. In vivo implantation demonstrates a four-week presence of these cells within the callus. Finally, FMA was successfully applied to a bone defect model, providing an opportunity for future investigation.
With a picture of the population identity, prepared with the knowledge of their capacity to induce healing, we can effectively investigate the fate of these cells in a model of fracture healing. The final goal was to begin to provide evidence as to how defects are affected by potential treatments.

5.1 In Vitro Identity

Our first goal was to meticulously characterize the isolated populations of EPCs and MSCs that were previously applied to an animal model. Our results demonstrate that both populations of cells meet many of the standards used in the literature to define the “typical” cell type. More importantly, it provides us with a standard population when applying these cells in in vivo models.

Our results show that greater than 95% of EPCs co-express cell-surface markers CD34, CD133, and VEGFR2. Functionally, EPCs bind to lectin, uptake AcLDL. The markers chosen for this study are based on a number of previous studies that have described the presence of CD34 and VEGFR2, AcLDL uptake and lectin binding, and the formation of tubule-like structures on Matrigel (Doyle, Metharam, and Caplice 2006; Boyer et al. 2000). In the strictest sense, a “true” EPC is one that is capable of self-renewal and differentiation into endothelial cells in vivo (Hirschi, Ingram, and Yoder 2008). To date, no single defining characteristic has been established to identify a true EPC (Hirschi, Ingram, and Yoder 2008). EPCs also share their defining surface markers with hematopoietic stem cells,
another rare cell type found in locations EPCs reside in, further complicating the ability to fully define this cell population (Risau 1995; Flamme and Risau 1992).

Since the inaugural report published by Asahara (1997), there have been a number of methods established to isolate EPCs (Boyer et al. 2000; Shi et al. 1998; Quirici et al. 2001; Peichev et al. 2000). Additionally, investigation into the culturing process has prompted the field to differentiate between EPCs that form early on in the culturing process (4-10 days in culture; early EPC or circulating angiogenic cell) and later forming EPCs (11-24 days in culture; late EPC or outgrowth endothelial cell) (Sieveking et al. 2008). Research suggests that the early EPCs function as angiogenesis inducing cells, producing an angiogenic effect through a paracrine mechanism. In contrast, late EPCs have been shown to engraft into nascent vasculature (Sieveking et al. 2008).

For the purposes of this study we have utilized a population of “early EPCs” to ensure we can relate this population to the healing capacity demonstrated in our previous investigations. The disparity between mechanisms, cell types, methods of isolation, tissue of isolation, and defining characteristics creates substantial variability in the literature. Therefore, it is reasonable to say that EPCs encompass a group of cells that exist in a variety of stages encompassing hemangioblasts to mature endothelium. As long as consistency is maintained between studies with regards to isolation and culture, the development of a therapeutic protocol is achievable.
In contrast to EPCs, MSCs have a more established definition developed by the International Society for Cellular Therapy (Dominici et al. 2006). The isolation and culture protocol met most of the requirements put forth by this paper. One benchmark missing from our study is flow cytometry data indicating a lack of expression of certain markers including CD11b, CD14, CD45, and HLA-DR. Despite this, we feel confident with the purity of our population given that 95% of cells in culture express all three of the positive markers. The importance of this result is identical to that of the EPCs. A standardized look at a series of studies can be achieved with a defined population associated with an isolation/culturing protocol. Investigation from our group has compared the effectiveness of EPC and MSC therapy in the critical-sized defect model. Evidence was provided that EPCs were superior to MSCs in our model. Furthermore, MSCs had little impact on the course of healing, a result that does not follow the trend in the literature. The results of the current investigation affirm that an appropriate population of MSCs were used in that study.

5.2 *In Vitro Potential*

The second aim of this study was to further characterize EPCs and MSCs based on their potential to incorporate into nascent tissue. Incorporation into fracture callus would mean differentiation into one of the components of fracture healing; osteocytes, chondrocytes, fibroblasts, or endothelium. Examining these results will give insight into the long-term fate of implanted cells in the callus.
5.2.1 EPC-Mesenchymal

The traditional model of EPC action is to induce an intensification of vessel formation. Depending on the population of EPCs isolated, vessel formation can be augmented by angiogenesis through paracrine actions or vasculogenesis through direct differentiation into endothelial cells. With endothelial potential established in ischemic disorders, it is easy to believe that the mechanisms would translate with the application to orthopaedic disorders. However, previous evidence suggests the existence of osteogenic properties from similar populations (Fadini et al. 2012). This raises questions as to the exact mechanistic actions of EPC implantation.

In pursuit of determining mesenchymal potential, EPCs underwent the same assay that determined the mesenchymal potential of MSCs. We show here that cultured endothelial progenitors express a low level of osteocalcin, a characteristic marker of osteoblast activity. Therefore, we have demonstrated a BM CD34⁺/CD133⁺/VEGFR2⁺ population with angiogenic properties in addition to an osteogenic marker. This is similar to the findings by Chen et al. (1997) who identified osteoblast precursor cells in cultured human BM CD 34⁺ cells (Chen et al. 1997). Large increases in post-differentiation expression of osteocalcin demonstrate a similar osteogenic capacity when compared with MSCs. It is difficult to phenotypically differentiate osteogenic EPCs and MSCs.
When examining the possibility that EPCs had equal capabilities compared to MSCs, we were able to demonstrate some key differences. Initially, we found that we were unable to determine the status of adipogenic differentiation due to the presence of the characteristic marker in both undifferentiated and differentiated populations. FABP-4, or fatty acid binding protein 4, is part of a family of small intracellular proteins that bind to a number of hydrophobic molecules in a number of different tissues (Zimmerman and Veerkamp 2002). It is a molecule that has very recently demonstrated to be expressed in endothelial cells in many tissues although expression in bone marrow was not examined (Elmasri et al. 2009). The same group went further to determine that FABP-4 expression is an important pro-angiogenic factor, acting as a target of VEGF as well as a modulator of cell proliferation (Elmasri et al. 2012). Ultimately, it becomes an interesting target for characterization without informing us to its adipogenic potential. There is currently a lack of evidence in the literature regarding adipogenesis and EPCs.

Secondly, EPCs lack the capacity to differentiate into chondrocytes in conditions where MSCs could. From this, we gather that the lack of full mesenchymal stem-ness may be either an inherent property of the EPC or that the combinations of inducing factors favored certain transitions in different cell types.

Recently, a number of groups have reported a phenomenon existing in vivo known as an endothelial-mesenchymal transition (EndMT). Medici D et al. (2010) describes the mechanisms of such a system based on the clinical pathology of Fibrodysplasia Ossificans
Progressiva (FOP) causing heterotopic ossification. The disease, characterized by a heterozygous mutation in the activin-like kinase 2 (ALK2) gene that encodes a receptor for BMP and TGF-β, causes endochondral ossification of chondrocytes of an unknown origin. What Medici D et al. (2010) was able to show was the presence of endothelial markers on chondrocytes and osteoblasts removed from one such ossification in addition to the induction of mesenchymal transition by endothelial cells following administration of TGF-β2/BMP-4 (Medici et al. 2010).

These finding raise questions as to whether endogenous levels of the osteogenic growth factors stimulate endothelial transformation in vivo. In situations similar to the defect model utilized later in the study, it is possible that the injury-stimulated release of growth factors induces a number of EPCs to undergo transdifferentiation. In the context of in vitro study it describes a pathway utilizing specific growth factors. Specialized induction pathways may be the reason for the lack of chondrogenic differentiation.

5.2.2 MSC-Endothelial

The traditional model for MSC fracture therapeutic mechanism is the bulk administration of osteogenic precursors leading to the formation of new bone at a faster rate. With the shifting paradigm of fracture repair moving towards the importance of vascular regeneration, the hope of MSC therapy influencing angiogenesis became an interesting area of investigation. Recent studies have hypothesized a role for MSCs in a number of disease
models including stroke (Chopp and Li 2002), diabetes (Lee et al. 2006), myocardial infraction (Kinnaird et al. 2004; Kocher et al. 2001), and hind-limb ischemia(Iwase et al. 2005; Kadiyala et al. 1997). Additionally, MSCs have functioned as stromal cells in solid tumors (Karnoub et al. 2007) and vascular stromal cells in wound healing models (Wu et al. 2007). The mechanisms reported include the release of cytokines/growth factors that promote endogenous angiogenesis (Kinnaird et al. 2004; Kadiyala et al. 1997; Iwase et al. 2005) as well as the direct differentiation into endothelial cells (Lee et al. 2006; Iwase et al. 2005; Wu et al. 2007).

We reported the angiogenic potential of MSCs based on a Matrigel tube formation assay. This assay allowed us to visually determine whether tube formation occurred in addition to quantification of the number of tubes formed. Quantification gave us the ability to crudely compare angiogenic potential between the undifferentiated progenitor cell populations and the endothelial nature of our differentiated MSCs. We were able to show the difference in tube formation capacity of MSCs increase significantly after exposure to EPC media. In fact, undifferentiated MSCs very rarely form tubes while differentiated MSCs form tubes in statistically equal amounts compared to EPCs. This highlights the idea that MSCs can be induced to show angiogenic properties similar to that of EPCs. This finding is significant because if an in vivo study found that MSCs were not participating in angiogenesis in any significant way, perhaps this type of transdifferentiation would increase the effectiveness of MSC therapy. Ultimately, in vitro potential of a single population gives insight into mechanism, whether transdifferentiation takes place or not.
Previous groups have also been able to stimulate these angiogenic capabilities *in vitro*. Oskowitz *et al.* (2011) demonstrated that under serum-deprived conditions, MSCs became angiogenic. They were able to produce MSCs that had a number of proangiogenic effects as well as phenotypic characteristics. The proangiogenic effects are illustrated by conditioned media experiments that contain angiogenic growth factors and stimulation of neovascular sprouts in chick aortic ring assays. Evidence for transdifferentiation are up-regulated angiogenic genes, expressed endothelial markers, and enhanced microtubule formation (Oskowitz *et al.* 2011). This study highlights that MSCs do have a potential that may or may not be realized *in vivo*.

### 5.3 *In Vivo* Cell Implantation

The second goal of this study was to determine the *in vivo* fate of implanted progenitor cell populations within the callus. Our results confirmed the presence of EPCs and MSCs up to 4 weeks *in vivo*, although conclusions regarding precise fate cannot be made. As mentioned previously, studies from our group have demonstrated the ostogenic/angiogenic nature of EPCs and the relative ineffectiveness of MSCs comparatively. The evidence presented here gives clues as to the reason behind the exhibited effect, although at this moment increased group numbers and additional assays are needed to confirm this relationship. Furthermore, longer duration studies are needed to examine whether the cells within implanted animals are becoming part of the ultrastructure of newly formed bone or whether the cells only remain in a transient state that supports future osteoid formation. The achievement garnered here is the development of an effective model to determine a long-term fate within the callus.
Fate of implanted cells is not limited to morphology, but location within the recipient tissue treated. We elected to use a non-bioactive scaffold to isolate the therapeutic potential of implanted populations. One result is that there is little control in the movement and health of the implanted populations. A possible result of implantation that would support healing models is a transient release of growth hormones/cytokines that induce tissue growth followed by either apoptosis or movement into circulation. It appears from samples that a large number of cells remain within the callus area. However, there is no reliable way of determining with the methods used the ratio of recently implanted cells to cells remaining after two and four weeks post-treatment. A technique such as radioactive labeling is needed to determine the number of cells alive after four weeks and theoretically beyond to complete healing at ten weeks. Live animal cell tracking will also help determine if there are a significant number of cells travelling to other organs. It is important to recognize the importance of finding a large number of cells within the callus at four weeks for both groups, supporting the idea of engraftment in the bone-healing model.

The time points used for this project were chosen for the lack of calcified bone formation that would increase the difficulty in sample processing. This preliminary success encourages us to pursue a number of simple decalcification techniques that would allow visualization of fate through complete remodeling. Another benefit that decalcification will allow is to excise the proximal and distal ends of the fracture site to attain a more complete picture of the tissue. The current study, limited by the lack of decalcification, created a situation where it was difficult to examine the tissue in a standardized way. Despite further work being needed on the model, there are several observations that can be made.
5.3.1 Control Implant

The first observation that can be made is when comparing the progression of healing between control and treated two-week groups. Notable differences include the presence of residual hematomas within the control groups. These structures are not present in either EPC or MSC calluses. The hematoma is responsible for the release of large amounts of cytokines and growth factors that aid in processes like angiogenesis. This is evident from studies that have removed the hematomas from fractures resulting in significantly attenuated calluses (Carano and Filvaroff 2003). Both EPC and MSC populations have been previously demonstrated to be potent factories of secretory factors (Miyamoto 2007; Salem and Thiemermann 2010). Therefore, one possible explanation for the result would be that the addition of the cell populations increase factors that induced hematoma degradation at an accelerated pace.

5.3.2 Cell Implant

EPCs and MSCs have been visualized in different aspects of the fracture callus in a variety of morphologies. It is interesting to note that EPCs cannot be clearly identified around discernable blood vessels. This fact alone suggests the observed healing capacity noted in previous studies is accomplished by either paracrine angiogenic or direct osteogenic mechanisms. The literature on EPC mechanisms in other ischemic healing models suggests the mode of healing can be paracrine in nature with certain populations. Retention rates of EPCs in targeted organs have ranged from 0% to 80% (Urbich 2004; Shantsila, Watson,
and Lip 2007), while a number of studies have shown less than significant numbers of EPCs within the inner lining of vessels (Purhonen et al. 2008; Rajantie et al. 2004). These studies combined with the results demonstrated here suggests that vascular engraftment in bone differs little from other tissues.

Recently, Kawakami Y et al. (2012) published their own report of EPC transplantation in a fracture model. One conclusion that this group made was that implanted EPCs possess the ability to undergo differentiation into both osteogenic and endothelial lineages \textit{in vivo}. In addition to the evidence of direct differentiation, they were able to demonstrate a total increase in osteogenic and angiogenic factor release within the callus as a whole (Kawakami et al. 2012). Furthermore, evidence exists that shows that EPC implantation \textit{in vivo} leads to an upregulation of BMP and VEGF, lending further credence to paracrine mechanisms (Li 2009). Although the models used to simulate fracture repair differ between studies, there are some important clues about shared mechanisms between implanted populations.

At these early time points and without additional staining, it becomes difficult to make any definitive conclusions as to the exact cell type that transplanted cells differentiate into. There are a number of further analyses that can be done in future experiments that will demonstrate with more confidence the precise identity of administered cells.
An important goal of future studies is to be able to determine fate of an implanted cell by histological structure (i.e. characteristic osteocyte morphology and location). Further identification techniques that involve immunohistochemical staining for a number of cell-surface markers can be a useful tool in determining the changes that occur in individual cells. One important investigation would be to examine the endothelial nature of implanted EPCs. Immunohistochemical staining for both endothelial and osteogenic markers would allow for more definitive conclusions pertaining to cell fate.

It is difficult to make any conclusions based on observations of MSCs implanted in a segmental defect. Like EPCs, MSCs are found in various areas of the callus at both two and four week time points. These results confirm that the absence of healing seen from MSC therapy in our previous studies was not due to the lack of cell survival. Morphology of the cells in addition to the precise fate remains ambiguous at this time. Like EPC implanted groups, additional immunohistochemical tests need to be carried out to determine the identity of cells in vivo. Later time point investigations will be necessary to determine the true fate of implanted cells.

As previously described in this study, we have found that under certain conditions MSCs can be coaxed into becoming endothelial in nature. It is also known that MSCs can function as endothelial supporting cells called pericytes (Crisan et al. 2008). These observations have given credence to the concept that MSCs can assist in healing by a mechanism other than direct osteoblast or chondrocyte differentiation. We have also been able to show evidence within the sample tissue to support endothelial MSCs. MSCs were observed
lining blood vessels in histological sections. However, this result is rare from the samples that were taken. There are a couple hypotheses that can be made to explain this result. First, this observation happened by chance and is a mere artifact of implantation into a vascularized tissue. Second, there is a subset of angiogenic cells within this population, in spite of flow cytometry data suggesting a homogeneous population based on the markers examined. Finally, it is possible that there was a condition within this sample that induced transdifferentiation into endothelial lineage. More in depth examination is needed to deduce the nature of this finding. Because this result is rare, it is likely that MSC therapy has an insignificant impact on fracture vascularity and therefore does not contribute to healing in this respect.

5.4 Fluorescent Microangiography

The technique of FMA undergone here was established based on protocols utilized by Dutly AE et al. (2006) for visualization of pulmonary vasculature (Dutly et al. 2006). The methods described were able to define the progression of pathology related to pulmonary hypertension. This technique was further utilized within investigations related to the renal microvasculature and the pathology related to renal disease (Advani et al. 2011). These groups felt it was necessary to develop a novel method for visualizing and quantifying nascent vasculature due to the shortcomings of the established methods. The value garnered with FMA is a procedure capable of discerning functional vasculature within the hind-leg of the animal.
The rationale for utilizing this procedure in an orthopaedic setting is two-fold. Firstly, with FMA, we can link the transplant therapy to vessel formation. Previous studies have indicated increased blood flow with laser Doppler analysis and capillary density based on histological techniques (Nauth 2012). Although each technique has merit, they are limited and certain conclusions are difficult to make. Important information pertaining to qualities of the nascent vasculature cannot be drawn from those data.

Secondly, the development of methods to analyze callus vasculature in a more precise or accurate approach is important to a field where bone vascularity and healing has been demonstrated to be inextricably linked. The time point of two weeks was chosen as a result of the revascularization process that peaks during defect healing. It is also convenient due to the lack of calcification that allows for tissue sectioning with less processing. We were able to demonstrate proof-of-concept with this technique as well as generate preliminary data to begin to elucidate the differences between groups.

Our group was not able to determine a significant difference in either total vessel length or total vessel volume. However, there does exist a trend towards increased vessel length and volume in the EPC implanted group versus both MSC and control groups. Because of the preliminary nature of this study, the n-value in each group remains low. The small group sizes make it difficult to determine significance. Therefore, to construct any important conclusions from this data, more studies will need to be conducted. Increased vessel density in the EPC group would concur with previous results.
The reconstructed images of vascular networks at this point in the study does not allow us to make any conclusions. We have shown with this technique that we can create 3D representations of the vascular network. Further studies are needed to fully assess the potential conclusions that can be drawn from these images. Factors such as network structure and vessel quality may be more predictive of healing. Blood flow increases seen in other studies can suggest a bulk increase in vessels or a functionally better network capable of carrying more blood.

An additional limitation to both FMA as well as the histological examination of the fracture sites are the non-standardized sampling of the callus at various points throughout the callus. More specifically, there is potential for variation of vasculature between samples taken from callus located closer to the ends of the bone versus a sample taken towards the center of the callus. Future studies are needed to refine the protocol to adequately sample different areas of the defect site.
6 Conclusion

The first aim of this study was to characterize progenitor cell populations isolated for the purpose of bone-defect healing. Isolated MSCs were found to match established definitions of MSC populations in the literature. Similarly, EPC cultures examined by multiple assays matched characteristics provided by the literature. The results allow us to link phenotypically described populations to cell-based therapies.

The second aim of this study was to characterize the functional potential of progenitor cell populations in mesenchymal and endothelial lineages. As expected, MSCs were able to differentiate into the three mesenchymal lineages. Our results demonstrate the in vitro ability of EPCs to undergo osteogenic differentiation, however, without the ability for chondrogenic differentiation and an inconclusive ability for adipogenic differentiation. In vitro angiogenic ability of EPCs was confirmed with a tube formation assay and shown to be far superior when compared to MSCs. These observations may explain why EPCs, with the possibility to target both bone formation and vascularity, were shown to be more effective in our defect model when compared to MSCs. Interesting observations were made when MSCs were subject to EPC media with regard to increased tube formation. The significance of these results is unknown at present.

The third aim of this study was to investigate the fate of implanted progenitor cell populations in a rat femur critical-sized defect model. We were able to demonstrate a
proof-of-concept study that will allow future investigation into the precise fate of autologous cells. We have shown that implanted EPCs and MSCs remain in the fracture site at both two and four week post-surgery, engrafting into the callus tissue.

In addition to the fate of implanted EPCs and MSCs, an examination into the consequences to nascent vasculature was carried out. We have successfully applied a technique that is novel to orthopaedic settings. Although the differences in vasculature were found to be non-significant, trends in favor of EPC therapy were demonstrated and more investigation is needed to discern true differences between transplanted groups.
7 Future Directions

The ultimate goal of investigations such as this is to promote advancement of novel therapies to treat orthopaedic disorders. The conditions set out were used to study a basic autologous cell therapy for treatment of bone defects that otherwise would fail to heal. EPCs or MSCs are taken from the patient, seeded to a non-bioactive scaffold carrier, and administered directly to the site of a nonunion during surgical intervention. This is the basis for CBT.

Barriers impeding the translation from preclinical models to trials in human patients still remain. In order to garner support for clinical trials in many diseases, the scientific community and regulatory bodies have to be satisfied with certain evidence obtained through preclinical study. Precise identification and mechanisms of action are among the types of evidence needed.

We believe we have accomplished our goal with the thorough set of characterization assays in populations of cells that have been previously linked to fracture healing. Further molecular and cellular studies into the functional aspects of transdifferentiation may shed light onto a highly debated topic. Although cells in an in vitro state may not behave in a similar manner, it becomes important to define the conditions that will induce these alternate states and functions. In vivo demonstrations of mechanism remain the gold standard of evidence of true fate.
It is a technically challenging task to precisely locate implanted cells in an animal model. It becomes more difficult to determine their function based on a histological preparation. We believe it is an accomplishment to establish a model to demonstrate this evidence. This study has presented preliminary data from a model with the potential to discern much more. There are a number of improvements to the technical process that should be investigated.

Initially, callus sectioning post-excision needs to be standardized following optimization. This task could be accomplished with the development of a decalcification protocol that retains antigenic integrity. The three benefits of this are: (1) the ability to visualize the perifracture response, (2) the establishment of visual plane to accurately section the callus to obtain a view of the entire callus, and (3) to extend the time-points past the four-week mark. Points 1 and 2 refer to altering cutting methods to obtain longitudinal sections that extend from the proximal end of the fracture distally. Following the defect for a longer period of time would provide a more comprehensive understanding of mechanism. After improvements are made to the model, further immunohistochemical stains related to the identity of implanted cells would aid in modeling mechanisms of action.

An assumption made at the onset of this study was that a majority of implanted cells remain at the fracture site. We believe that our assumption was justified upon viewing the large amount of cells stained within the treated specimens. However, it is difficult to fully prove this assumption with techniques that we have used. A further cell tracking study that
involves the *in vivo* tracing of implanted cells would allow for real-time following of the cells throughout the early stages of healing.

The final aspect of this study was the novel application of FMA to a fracture callus. Angiography provided interesting preliminary data pertaining to the quantity of vasculature at the fracture site. Increasing the size of the groups will allow for a better determination of significance. Utilizing more 3D models of the vasculature will hopefully yield further data and potential conclusions regarding the effects of CBT on qualities of the nascent vasculature. Finally, adding fluorescently tagged cells to the model could theoretically allow for a large-scale visualization of relationships between implanted cells and vessels.

In addition to the studies presented in this report, there are a number of variations in the application of CBT that have yet to be explored. Included in this group is the co-implantation of EPCs and MSCs as well as the use of a variety of more bioactive scaffolds. Furthermore, movement to large animal studies to assess effectiveness will provide more evidence towards clinical application. Overall, the growing body of literature on orthopaedic tissue engineering illustrates the vast potential for these investigations to improve the lives of real patients in the near future.
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