Endothelial Progenitor Cells (EPCs) for Fracture Healing and Angiogenesis: A Comparison with Mesenchymal Stem Cells (MSCs)

by

Aaron Nauth, MD, FRCSC

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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2012

Abstract

The purpose of this study was to compare the effects of two types of stem/progenitor cells on the healing of critical sized bone defects in a rat model. Endothelial progenitor cells (EPCs), a novel cell type with previously demonstrated effects on both osteogenesis and angiogenesis, were compared to both a control group (no cells), and a treatment group of mesenchymal stem cells (MSCs). The hypothesis was that EPCs would demonstrate both superior bone healing and angiogenesis, when compared to MSCs and controls. EPCs, MSCs, or a control carrier were placed in surgically stabilized bone defects in a rat femur and both bone formation and angiogenesis were assessed. EPC treated defects demonstrated significantly more bone formation and angiogenesis at the bone defect site than MSC or control treated defects. These results strongly suggest that EPCs are more effective than MSCs for therapeutic osteogenesis and angiogenesis in a bone defect model.
Acknowledgments

First and foremost, I would like to acknowledge my supervisor Dr. Emil Schemitsch for his guidance, mentoring, and friendship throughout both this graduate degree and my orthopaedic career. His dedication to education, research, and patient care in orthopaedic surgery is truly an inspiration. Second, I would like to acknowledge Dr. Ru Li for his help and technical expertise with this project. Third, I would like to thank Dr. Jane Aubin and Dr. Cari Whyne for their invaluable contribution to this project as members of my program advisory committee. Finally, I would like to thank my wife, Rhonda, for putting up with all of those hours spent in the lab or on-call. Of all the blessings I have been granted in life, she is by far the greatest.
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<tr>
<td>EPCs</td>
<td>Endothelial Progenitor Cells</td>
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<td>MSCs</td>
<td>Mesenchymal Stem Cells</td>
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<tr>
<td>BMPs</td>
<td>Bone Morphogenetic Proteins</td>
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<tr>
<td>AICBG</td>
<td>Autogenous Iliac Crest Bone Graft</td>
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<tr>
<td>PDGF</td>
<td>Platelet Derived Growth Factor</td>
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<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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<tr>
<td>FGF</td>
<td>Fibroblast Growth Factor</td>
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<tr>
<td>microCT</td>
<td>Micro-Computed Tomography</td>
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<tr>
<td>EBM-2</td>
<td>Endothelial Basal Medium</td>
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<tr>
<td>hEGF-β</td>
<td>Human Epidermal Growth Factor-β</td>
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<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<td>R3-IGF-1</td>
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<td>hFGF-β</td>
<td>Human Fibroblast Growth Factor</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>FLK-1</td>
<td>Fetal Liver Kinase-1</td>
</tr>
<tr>
<td>vWF</td>
<td>Von Willebrand Factor</td>
</tr>
<tr>
<td>PLP</td>
<td>Para-Formaldehyde</td>
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</table>
AP: Antero-Posterior
Kv: Kilovolts
mAs: Milliamps
3-D: Three Dimensional
ROI: Region of Interest
BV: Bone Volume
BV/TV: Bone Volume Density
Conn D: Connectivity Density
BS: Bone Surface Area
BS/BV: Bone Surface Area to Bone Volume Ratio
Tb.N: Trabecular Number
Tb.Sp: Trabecular Spacing
LDPI: Laser Doppler Perfusion Imaging
LDF: Laser Doppler Flowmetry
SE: Standard Error
ANOVA: Analysis of Variance
DAPI: 4',6-diamidino-2-phenylindole
Ang-1: Angiopoietin-1
SDF-1  Stromal Cell-Derived Growth Factor-1

GM-CSF  Granulocyte-Macrophage Colony-Stimulating Factor
Chapter 1: Literature Review

1.1 Clinical Relevance

The management of bone defects and nonunions (non-healing fractures) continues to represent a significant clinical challenge for orthopaedic surgeons. The scope of this challenge is quite large with an estimated 15.3 million fractures occurring annually in the United States alone, 5 to 10% of which are complicated by impaired healing.\textsuperscript{1,2} The current treatment of bone defects and nonunions often requires multiple surgical procedures, prolonged hospital stays, and long periods of non-weight-bearing through the affected extremity. This results in significant patient morbidity as well as prolonged periods of disability. In extreme cases, bone defects or nonunions refractory to current treatment options can require amputation of the affected limb. Due to the significant burden of impaired fracture healing, a potential solution to this difficult problem would impact a large number of patients and relieve a substantial socioeconomic burden.

Bone defects may arise from trauma, infection, or tumor. Bone defects in orthopaedic trauma patients typically arise in 1 of 2 ways. In the first, patients with high energy open fractures may present with bone loss secondary either to the acute loss/extrusion of bone at the time of
injury or the removal of contaminated bone during surgical debridement (Figure 1). These injuries occur due to high-energy trauma with associated injury to muscle, soft tissues, and blood vessels occurring concomitantly with the bony injury. Injury to these associated tissues results in significant impairment of vascularity at the bone defect site.
Figure 1: Radiograph following surgical debridement of a high-energy open tibia fracture in a 33 year old male patient. The bone defect has been filled with antibiotic cement beads to act as a temporary spacer and prevent infection while the patient awaits reconstruction of the bone defect.
The second clinical scenario in which bone defects present in orthopaedic trauma patients is that of nonunions. A classification of nonunions has been described which categorizes nonunions into hypertrophic, oligotrophic, and atrophic. The classification of nonunions into one of these three categories has clinical implications for treatment. Hypertrophic nonunions present with abundant callus formation at the fracture site but failure of healing due to excessive motion at the fracture site. In this setting the introduction of biomechanical stability through internal fixation reliably results in healing of the fracture. Oligotrophic nonunions present with some callus formation but failure of fracture healing due to a combination of excessive motion and impaired biology at the fracture site. Treatment in this scenario is directed at increasing biomechanical stability and augmenting fracture biology (typically with autogenous bone grafting). The third, and most difficult to treat, type of nonunion is atrophic nonunions. In this setting the bony injury has failed to heal due to a number of potential factors including infection, initial high-energy injury, multiple surgeries, or patient comorbidities (such as smoking, diabetes, or malnutrition). Atrophic nonunions present not only with failure of the bone to heal, but also resorption of the bony ends of the fracture, resulting in a bone defect (Figure 2). Due to the contributing factors associated with atrophic nonunions, these bone defects, like high-energy open fractures, also present with impaired vascularity. The
treatment of atrophic nonunions requires dedicated efforts to restore biology and vascularity at
the fracture site.

The impaired vascularity that accompanies bone defects secondary to open fractures or
nonunions has long been recognized as a significant issue. More recently, the importance of
angiogenesis (the formation of new blood vessels) as a critical process for the healing and
regeneration of bone has been recognized, particularly in the setting of bone defects. Bone
is a highly vascularized tissue and requires a close coupling between blood vessels and
osteogenic cells to maintain skeletal integrity. As such, it is becoming increasingly clear that
strategies directed at the regeneration of bone should also incorporate strategies for enhancing
vascularity. At the current time there are limited clinical options available to orthopaedic
surgeons for enhancing vascularity at sites of desired bone regeneration.
Figure 2: Radiograph of a 74 year old female patient with an atrophic nonunion of her femoral shaft. The patient has had multiple surgical procedures and presents with failed hardware and an unhealed fracture.
1.2 Critical Components of Fracture Healing

It is important to recognize that in addition to vascularity, there are a number of critical components required for the healing and regeneration of bone (Figure 3).13

1. **Osteogenesis** refers to an appropriate population of osteoprogenitor cells to participate in the bone-forming process. The popularity of mesenchymal stem cells (MSCs) for cell-based therapies for fracture healing is due to their known ability to differentiate into these cells.

2. **Osteoconduction** refers to an appropriate scaffold to support the migration and attachment of osteoprogenitor cells and blood vessels.

3. **Osteoinduction** refers to signaling of the bone-healing cascade by various cytokines and growth factors. The bone morphogenetic proteins (BMPs) are the most widely studied group of osteoinductive proteins (their signaling cascade is depicted in Figure 3).

4. **Mechanical Stability** refers to appropriate stability of the bony ends of a fracture and is necessary for fracture healing to occur.

5. **Vascularity** at the fracture site and soft tissues surrounding the fracture is a critical component of fracture healing. There is a paucity of pre-clinical data on therapies which
promote vascularity at sites of desired bone healing and effective clinical therapies for enhancing fracture vascularity are lacking.

Figure 3: Illustration of the critical components of fracture healing.

In the presence of the above elements, fracture healing follows a reliable cascade of events that restores the bone to its pre-injury state. The initial stage involves hematoma formation from disruption of the local endosteal and periosteal blood supply. A subsequent inflammatory stage occurs, characterized by the release of inflammatory mediators from local cells and platelets. This results in a progressive increase in blood flow
that peaks approximately 2 weeks after fracture. The subsequent revascularization phase is characterized by the formation of new blood vessels by endothelial progenitor cells, which arrive via the bone marrow and circulation. This is followed by the infiltration and proliferation of mesenchymal stem cells, which then differentiate based on the local conditions of the fracture site.

The degree of stability (inversely related to strain), in combination with the oxygen tension and signals from local growth factors, influences the type of fracture healing that occurs, primarily via their effects on the differentiation of local progenitor cells. High strain with low oxygen tension promotes the differentiation of cells that produce fibrous tissue, leading to fibrous non-union. Low strain and high oxygen tension tend to promote the formation of woven bone directly (hard callus), through a process referred to as intramembranous bone formation. Regions of intermediate strain and low oxygen tension promote chondrocyte differentiation and cartilage formation (soft callus). The cartilage callus stabilizes the fracture site, reducing strain and allowing the calcification of the cartilage matrix and bone formation by osteoblasts in a process known as enchondral bone formation. Enchondral and intramembranous bone formation occur in fractures with varying degrees of relative stability. Relative stability occurs clinically in the setting of
fractures stabilized with intramedullary nails (load-sharing devices) or bridge plates. A third type of fracture healing occurs in fractures with cortical contact and direct compression (absolute stability), such as might occur in a situation of a humeral shaft fracture, anatomically reduced and stabilized with a compression plate. In this setting primary healing occurs via direct haversian remodeling, whereby osteoclasts resorb bone and create cutting cone channels across the fracture site allowing blood vessels and osteoblasts to follow.

Fracture healing progresses through the repair phase, usually with a combination of intramembranous and enchondral bone formation. The final phase involves remodeling of the immature woven bone to mature lamellar bone, which occurs over months to years.13
1.3 Autogenous Iliac Crest Bone Graft (AICBG): The Current Gold Standard

The current gold standard of treatment for bone defects and nonunions is grafting of the defect with autogenous iliac crest bone graft (AICBG). AICBG possesses a viable population of osteogenic cells, contains osteoinductive proteins (such as the BMPs), and has large amounts of cancellous bone which serve as an ideal osteoconductive scaffold. AICBG therefore addresses several of the critical components of fracture healing. AICBG is readily available from the patient’s own anterior or posterior iliac crest in limited quantities. This treatment has been widely reported in the orthopaedic literature for decades. However, there are several recognized shortcomings of AICBG. First, a separate incision is required with associated morbidity, surgical time, blood loss, and post-operative pain. Second, a number of complications may arise from harvest of the graft including infection, hematoma, nerve injury, iatrogenic fracture, hernia, and chronic pain. The reported incidence of complications from AICBG harvest ranges from 6-40%. Third, a limited volume of graft is available for harvest from the iliac crest, limiting the defect size that can be effectively treated with AICBG. Generally regarded limits to the extent of a bone defect that can be treated effectively with AICBG have been reported as 5-7 cm. Finally, and perhaps more importantly than the
aforementioned issues, AICBG can have a significant failure rate in the management of bone
defects and nonunions. As previously mentioned, multiple surgical procedures and
prolonged periods of non-weight bearing are often required.

1.4 Tissue Engineering for the Regeneration of Bone

The significant limitations of AICBG described above have lead to large field of research
exploring potential alternative treatments for the healing of bone defects and nonunions. In
fact an entire new field in orthopaedic surgery call orthobiologics has evolved in the last two
decades dedicated to the exploration of bone graft substitutes. Several bone graft substitutes
have been developed and investigated both in the preclinical and clinical setting. Currently
available bone graft substitutes include osteoconductive scaffolds such as calcium phosphates,
and osteoinductive proteins such as the BMPs and platelet derived growth factor (PDGF).
However, despite extensive investigation, including randomized control clinical trials, no bone
graft substitute has proved superior to AICBG in the challenging clinical setting of a bone
defect. Even combinations of osteoinductive proteins and osteoconductive scaffolds such as
allograft/BMP or calcium phosphate/platelet-derived growth factor (PDGF) have failed to
demonstrate clinical superiority over AICBG. Many authors have suggested that this is not
surprising given the fact that most of the currently available bone graft substitutes address only one or two of the critical components of fracture healing, whereas AICBG provides osteoconduction, osteoinduction, and osteogenesis. Two deficiencies that have been consistently identified in the literature with regard to the currently available alternatives to AICBG are lack of responding cells (osteogenesis) and lack of vascularity (angiogenesis). 

This has lead many investigators to explore more sophisticated strategies of tissue engineering in orthopaedics, which attempt to combine several of the critical components of fracture healing, in order to address the difficult problem of bone defects. Among the most successful have been cell-based approaches, which involve the use of a population of cells placed on an osteoconductive scaffold, often in combination with osteoinductive growth factors. The majority of investigators have employed the use of MSCs due to the known potential for these cells to differentiate into chondrogenic and/or osteogenic cells, the main cellular mediators of bone formation. Several investigators have demonstrated positive results in animal models with the use of MSC’s combined with an osteoconductive scaffold for the treatment of nonunions and bone defects. However, despite decades of pre-clinical research, there has been minimal clinical experimentation with MSC’s and several shortcomings have been identified. These shortcomings include limited viability of transplanted cells in large bone
defects, lack of osteoinductive stimuli, and, in particular, a lack of angiogenesis with MSC transplantation. In order to address these shortcomings, many investigators have used genetically modified MSC’s which express osteogenic and/or angiogenic proteins. These studies have demonstrated superior results of genetically modified MSC’s versus MSCs alone using multiple different proteins including BMPs, vascular endothelial growth factor (VEGF), and fibroblast growth factor (FGF). In fact, the best results have been demonstrated using a combination of osteogenic and angiogenic proteins, addressing both bone and blood vessel formation in single strategy. However both safety concerns and cost issues regarding the use of gene therapy for orthopaedic conditions have precluded the clinical investigation of genetically modified MSCs for bone regeneration, and to date no clinical trials of genetically modified MSCs have been conducted.

Primary among these impediments are safety concerns. Orthopaedic patients often represent a young, previously healthy population, and traumatic bone defects and non-unions, while representing extremely debilitating conditions with significant morbidity and socioeconomic impact, are ultimately non-fatal. This significantly skews the risk-benefit ratio of gene therapy’s application to orthopaedic trauma. There is significant trepidation regarding the application of gene therapy to non-fatal, non-genetic disorders in the clinical setting, and while these
concerns may represent an over-reaction to the perceived or theoretical risks of gene therapy, they cannot be dismissed. Viral vectors and their ability to cause dangerous immune reactions, insertional mutagenesis, or uncontrolled long-term expression of the transferred gene continue to be at the forefront of the scientific community’s apprehension towards gene therapy.\textsuperscript{51}

There has been one reported death and two cases of the development of leukemia-like conditions attributed to gene therapy using viral vectors for non-orthopaedic conditions.\textsuperscript{53}

More recently in 2007, an orthopaedic gene therapy trial for rheumatoid arthritis was halted after a patient died following an intraarticular knee injection of a viral vector encoding for etanercept.\textsuperscript{54} Ultimately, the patient’s death was attributed to a disseminated histoplasmosis infection and excessive internal bleeding. There was no clear evidence that the gene therapy was responsible and the trial was re-started. Ultimately, the concerns with viral gene therapy may be overcome by the use of non-viral methods for gene transfer. While many investigators feel that non-viral gene transfer may be safer, further research is needed to justify this position.

A further constraint of gene therapy and its application to fracture healing is cost. Ex vivo strategies, in particular, represent a time-consuming and expensive process due to the necessity of multiple stages of cell harvest, culture expansion, transduction/transfection, and reimplantation. A cell-based strategy that provides a combined targeting of osteogenesis and
angiogenesis, while avoiding the safety concerns and cost issues of gene therapy, remains desirable and largely uninvestigated.

1.5 Endothelial Progenitor Cells (EPCs)

Endothelial progenitor cells (EPCs) represent a population of novel progenitor cells, of hematopoietic origin, with known ability to participate in angiogenesis.\(^{55}\) It has been shown that EPCs home to sites of tissue ischemia, effect functional blood flow recovery in ischemic tissues, and enter the circulating system in response to trauma.\(^{56-59}\) The effectiveness of EPCs at inducing angiogenesis in ischemic tissues is well documented in animal studies in the areas of cardiovascular disease, peripheral vascular disease, and stroke.\(^{60-62}\) The remarkable success of EPC therapy in animal models of ischemia has led to clinical trials of EPC therapy for myocardial infarction and limb ischemia.\(^{63-66}\)

Given the documented ability of this cell population to therapeutically effect angiogenesis in ischemic conditions, their application to bone defects and nonunions (which exhibit impaired vascularity) seems intuitive. The investigation and therapeutic application of EPCs for bone regeneration is relatively novel. Several investigators have very recently reported success with
the application of EPCs to critical sized bone defects and non-union models in animal studies. Therapy with EPCs has been shown to augment both fracture healing and local angiogenesis in animal models of non-union. Moreover, EPCs have recently been shown to be capable of differentiating into osteogenic cells in vitro, and have been shown to be up-regulated in response to orthopaedic trauma in humans.

Based upon this recent evidence, EPCs appear ideally suited for the treatment of nonunions and bone defects, given their ability to target both osteogenesis and angiogenesis without the need for gene therapy. However, to date there has been no comparison of EPCs with the more commonly employed MSCs to confirm these potential benefits. In addition, the angiogenic capacity of these two cell types has not been compared in an animal model of the fracture healing. The current study sought to address this by comparing both the osteogenic and angiogenic capacity of EPCs versus MSCs in an animal model of fracture healing.
Chapter 2: Aims/Hypotheses

The central aim of the current study was to compare the effects of two stem/progenitor cell types (EPCs versus MSCs) on the healing of critical sized bone defects in a rat model. In addition, the secondary aim was to compare the effects of these two cell types on local angiogenesis at the site of bone regeneration. The central hypothesis was that EPCs would demonstrate superior bone healing and angiogenesis when compared to MSCs. The rationale for this hypothesis was based on the previously cited literature which has shown that MSCs, in the absence of genetic modification, are often insufficient to effectively regenerate bone due to their inability to stimulate angiogenesis at the site of the bone defect. In contrast, recent investigation has demonstrated the capacity of EPCs to stimulate both osteogenesis and angiogenesis in animal models of bone defects and nonunions. It was hypothesized that this combined targeting of osteogenesis and angiogenesis would lead to superior bone defect healing.

In order to test this hypothesis a series of research questions were posed and answered by relevant experiments:
1. Are EPCs superior to MSCs in an animal model of bone defect healing?

In order to assess the bone defect healing potential of EPCs versus MSCs, a critical sized bone defect was created in the femur of a rat. Following creation of the defect a carrier (gelfoam) was placed in the defect after seeding with an equivalent population of EPCs, MSCs, or no cells (negative control). Bone defect healing was then assessed radiographically with a combination of plain radiographs and micro-computed tomography (microCT). MicroCT allows an objective quantification of bone growth by comparing parameters such as bone volume and bone density between the treatment groups. In addition, an objective functional assessment of bone healing was carried out to compare the treatment groups, using biomechanical testing of the specimens after 10 weeks of healing was allowed.

2. Does local EPC therapy result in superior angiogenesis relative to MSC therapy and controls?

Angiogenesis was assessed in EPC treated, MSC treated, and control defects using a combination of histology and Laser Doppler to assess both blood vessel formation and
local blood flow. The advanced Laser Doppler imaging used in this study allows objective quantification of both bone and soft tissue blood flow.
Chapter 3: Methods

3.1 Experimental Design

The experimental design is depicted in Figure 4. Three experimental groups were created. In the control group a gelfoam sponge (collagen scaffold) with saline only was placed at the surgically created bone defect site. In the EPC group, the gelfoam sponge was seeded with $1 \times 10^6$ EPCs and then placed at the defect site. In the MSC group, the gelfoam sponge was seeded...
with $1 \times 10^6$ MSCs and then placed at the defect site. The number of animals in each group and
the harvest time points are shown in Table 1.

Animals harvested at 3 weeks were used for Laser Doppler Perfusion Imaging of soft tissue
blood flow and Laser Doppler Probe assessment of bone blood flow, as well as histology.

Animals harvested at 10 weeks all had microCT analysis of bone formation and Laser Doppler
Probe analysis of bone blood flow. Half (n=7) of the 10 week harvested animals in each group
were then used for biomechanical testing of the functional strength of the regenerated bone,
and the other half (n=7) were used for histology. All animals had plain radiographs performed
every 2 weeks. The time points for animal sacrifice were selected based on our previous
studies of this bone defect model.\textsuperscript{67, 72}

<table>
<thead>
<tr>
<th>Groups</th>
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<th>10 week sacrifice</th>
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</tr>
<tr>
<td>II. MSC</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>III. Control</td>
<td>3</td>
<td>7</td>
</tr>
</tbody>
</table>

Total Number = 51

Table 1: Specimens in each experimental group at different time points.
3.2 Cell Isolation, Culture, and Identification

EPCs were isolated from the bone marrow of syngeneic rats (Fisher 344) using the previously described method of differential culture. The animals were sacrificed at a standardized age (8-10 weeks) and weight (250-300 grams). Cells isolated from one animal were used for surgery on one animal with a bone defect. After sterile isolation of the femora and tibiae of donor rats, bone marrow cells were collected and isolated from the rat bone marrow by flushing the shaft with buffer (PBS/0.5%BSA/2mM EDTA; Invitrogen) using a syringe. The cells were subsequently disaggregated by gently pipetting 10-20 times. The cells were then passed through a 30 µm pre-separation filter to remove remaining clumps. The cells were then centrifuged and the supernatant removed. The cell pellet was resuspended in 4 ml of buffer. Bone marrow mononuclear cells were separated by the Ficoll-paque gradient centrifuge. The layer of mononuclear cells was carefully aspirated, resuspended with medium, and plated on to fibronectin coated tissue culture flasks. The cells were maintained in endothelial basal medium (EBM-2, Clonetics) supplemented with EGM-2-MV-SingleQuots, which contains 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 incubated at 37 degrees
Celsius in a humidified 5 per cent carbon-dioxide environment. After 4 days in culture, non-adherent cells were removed by washing with phosphate buffered saline (PBS), new media was applied, and the culture was continued for 7 to 10 days. For identification of EPCs, immunocytochemistry was used as previously described. Briefly, cells were incubated overnight at 4°C with the primary antibodies (CD34, CD133, FLK-1, and vWF; Invitrogen), counterstained with DAPI, then visualized under fluorescent microscopy. Previous investigation has demonstrated that this protocol results in a relatively homogenous population of EPCs, capable of forming tube like structures on Matrigel in vitro, indicative of angiogenic capacity.73,74

MSCs were collected in an identical fashion from the bone marrow of donor rats. After cell collection, the cells were suspended in MSC specific medium [fresh α-MEM supplemented with 10 percent fetal bovine serum, 2% penicillin G/streptomycin, and amphotericin B (0.25 microgram per milliliter); Invitrogen] and plated on non-coated tissue culture flasks. The cells were incubated at 37 degrees Celsius in a humidified 5 per cent carbon-dioxide environment. On the fourth day of culture, the non-adherent cells were removed along with the culture medium. Fresh medium was added to the adherent cells twice per week, and the cells were passaged on the eleventh day. Cells from passages 3 and 4 were used for transplantation to bone defects as previous studies have indicated that by the third passage a uniform population
of MSCs capable of osteogenic, adipogenic, and chondrogenic differentiation can be obtained.\textsuperscript{75} In order to confirm we had obtained an appropriate population of MSCs, cells from passage 3 were stained using immunocytochemistry for CD105, CD29, CD44, and CD90 (Invitrogen) and counterstained with DAPI.

### 3.3 Cellular Seeding of the Gelfoam Scaffold

On the day of surgery the cells (EPCs or MSCs) were detached from their culture flask using trypsin/EDTA (Invitrogen), washed, and centrifuged. Following cell counting, 1 X 10\textsuperscript{6} cells (EPCs or MSCs) were suspended in 0.1 ml of saline and placed on top of a sterile gelfoam sponge (5mm X 5mm X 5mm) contained in a 0.5 ml centrifuge tube. Cells were allowed to absorb/seed the sponge for a minimum of 1 hour at room temperature prior to surgical transplantation.

### 3.4 Animal Model

We have previously described a rat model of a critical sized femoral defect.\textsuperscript{67} The study strain of fisher 344 inbred male rats (250-300 g) were anesthetized with Isoflourane inhalation: 5% for
induction and 2% for maintenance. A lateral incision was made and the entire length of right femur was exposed. The entire diaphysis of the femur was exposed and an oscillating saw was used to create two diaphyseal osteotomies under irrigation with 0.9% sterile saline solution forming a 5 mm segmental bone defect in the middle 1/3 of each femur. A mini plate with 5 holes was used to fix the fracture, with 4 self-tapping 1.5 mm cortex screws: 2 proximal and 2 distal. Subsequently, a gelfoam sponge containing either saline (control), EPCs, or MSCs was then placed into the bone defect. All soft tissue layers were closed meticulously (vicryl 5-0), and the skin sutured intracutaneously (nylon 4-0). Immediately after the operation, full weight bearing and cage activity was allowed. All animal protocols were approved by the St. Michael’s Hospital Animal Care Committee.

3.5 Specimen Harvest

The animals were sacrificed at either 3 weeks or 10 weeks by exsanguination after induction of general anesthesia with 5% Isoflurane inhalation. Soft tissues were dissected from the operated femur and denuded femurs were kept within 4% Para-formaldehyde (PLP; Invitrogen) solution for 24 hours for fixation. Following this step, plates and screws were removed from the bone and the specimens that were taken from the animals in the 3 week groups (n=9) were
immediately put in 70% ethanol for histologic processing. The femurs from the animals that
were sacrificed at 10 weeks (n=42) were stored in 70% ethanol until quantitative microCT was
performed.

### 3.6 Evaluation of Bone Healing

**Plain Radiography:** Serial radiographs were conducted throughout the experiment from defect
creation to animal sacrifice to determine the features of bone healing on the antero-posterior
(AP) view, using standardized tube to leg distance, kilovolts (Kv) and milliamps (mAs) each time.
Radiographs were taken at 0, 1, 2, and 3 weeks for 3 week groups, and every 2 weeks for the 10
week groups. The films were evaluated by 2 blinded assessors to define the bone formation
process in the surgically created defects. Included in the features examined were the amount
of callus formed, osseous bridging of the gap, and corticalization of the regenerated bone. The
final 10 week radiographs were classified as not healed, partially healed, or completely healed
on the basis of these features.
MicroCT Evaluation: MicroCT evaluation was performed in all 10 week specimens after harvest. Following tissue fixation with PLP, the plates were removed with care taken not to disrupt the bone defect site. The specimens were then scanned with microCT to evaluate the amount and three dimensional (3-D) structural variation of new bone formation within the defect area. The specimens were placed in the standard position in the scanner, parallel and perpendicular to the scan plane to allow for objective quantification of bone formation. Three-D volumes were reconstructed and the region of interest (ROI) was set at the site of the segmental defect, and selected for analysis from the recognizable margins of the original defect. The new bone formation and mineralization in the ROI were then evaluated using the following structural parameters: bone volume (BV, mm$^3$), bone volume density (BV/TV, %), connectivity density (Conn D, mm$^3$), bone surface area (BS, mm$^2$), bone surface to bone volume ratio (BS/BV, mm$^{-1}$), trabecular number (Tb.N, mm$^{-1}$), and trabecular spacing (Tb.Sp, mm).

Biomechanical Testing: Torsional strength and stiffness were measured for 7 of the ten week specimens from each group (EPC, MSC, and control), as well as 7 contralateral intact femurs in order to compare the strength of the bone healing in each group to that of intact bone. Testing was performed using the MTS Bionix 858 (MTS Systems, MN, USA). Each femur was aligned
longitudinally to the axis of the MTS and potted in polymethylmethacrylate, with the gauge
length defined as the length between the two potting casings. Torque was measured during
the application of angular displacement (1°/second) until failure or to a maximum displacement
of 40°. The load displacement data were smoothed using a moving normal filter with an
averaging interval of 10. The maximum torque, maximum angular displacement, and torsional
stiffness to failure were calculated based on the generated load displacement data. Torsional
strength was defined as the maximum load sustained during loading, and the torsional stiffness
was defined as the slope of the line extending to the point of maximum sustained torque.

Histology: The specimens from the 10 week group animals (n=21) were decalcified within EDTA
(Invitrogen) solution following microCT scanning and were cut through lines that were 5 mm
beyond the osteotomy lines on both sides. The samples then were dehydrated and embedded
in methylmethacrylate. The paraffin blocks were cut into 5-µm sections and the prepared
slides were stained with hematoxylin and eosin. After staining, the slides were evaluated
qualitatively at 20x magnification for comparison of the 3 groups with regard to bone formation
and bridging of the bone defect site.
3.7 Evaluation of Angiogenesis

**Laser Doppler Perfusion Imaging (LDPI):** LDPI was used to evaluate blood flow in the soft tissues surrounding the fracture site in the EPC, MSC, and control groups at 1 hour, 1 day, 3 days, 1 week, 2 weeks, and 3 weeks post-operatively. After a 10 minute warming period on a warming blanket, both the operated and non-operated limbs were imaged in the animals while under anesthesia, using the moorLDI2 laser Doppler imager (Moor Instruments Inc., Denver, USA). The use of this instrument to measure soft tissue blood flow has been described both in fracture models and models of hindlimb ischemia. After images were obtained in triplicate, a region of interest (ROI) was set around the fracture site and an identical ROI was set in the contralateral, non-operated limb (Figure 5). Average blood flow value was then calculated from the respective ROI’s and expressed as a ratio of fracture ROI/contralateral ROI using moorLDI2 software. Average soft tissue blood flow was then calculated for all specimens in the EPC group at the specified time points and compared to the average values obtained in the MSC and control groups.
Laser Doppler Flowmetry (LDF): LDF (Periflux PF3, Perimed Instruments) was used to assess cortical bone blood flow at the bone defect site pre-osteotomy, post-osteotomy, and immediately prior to animal sacrifice at 3 and 10 weeks. The use of this instrument to measure cortical bone blood flow has been previously described in an animal fracture model.\textsuperscript{77,78} Measurements were obtained at 3 anatomic locations after surgical approach: proximal to the bone defect, at the bone defect, and distal to the bone defect (Figure 6). Blood flow measurements were obtained by placing the probe directly on the bone at the 3 anatomic locations in a standardized fashion following surgical approach. Measurements were recorded.
in a blinded fashion over a 30 second time period using LDF software and then an average value

**Figure 6:** Anatomic locations for LDF Measurements. 1 = proximal to bone defect, 2 = at bone defect, 3 = distal to bone defect
was calculated from the 30 second data. Measurements were taken at the specified anatomic locations immediately prior to bone defect creation, immediately following bone defect creation, and prior to bone harvest at 3 and 10 weeks. All measurements were then expressed as a ratio of the blood flow values obtained prior to bone defect creation. Values from each post-osteotomy time point were averaged for all specimens in the EPC group and compared to the MSC and control groups.

Histology: The specimens from the 3 week group (N=9) were dehydrated and embedded in methylmethacrylate. The paraffin blocks were cut into 5-µm sections and the prepared slides were stained with hematoxylin and eosin. After staining, the slides were evaluated qualitatively at 20X magnification for comparison of the EPC group with the MSC and control groups for the number of blood vessels visualized.

3.8 Statistical Analysis

Mean and standard error (SE) values were calculated for all statistically analyzed parameters. Plots of means were used to display data distribution and to visually assess the differences
between the study groups. The significance of the differences in means between groups were statistically tested by ANOVA, followed by a multiple comparison procedure (a post hoc analysis) of Tukey’s honestly significant difference test to correct for the inflated risk of type 1 error during the process of multiple comparisons. The confidence interval was set at 95% and a value of \( p < 0.05 \) was considered statistically significant. All statistical analyses were performed with SPSS Base 15.0 for Windows (© 2007 SPSS Inc. Chicago, IL, USA).
Chapter 4 : Results

4.1 Cell Culture and Staining

EPCs: Cell culture was observed daily under phase contrast microscopy to evaluate the morphology of cultured cells. After 3-4 days in culture on fibronectin coated flasks, colonies of EPCs appeared consisting of multiple thin, flat cells emanating from a central cluster of rounded cells.

![Image of EPCs after 7 days in culture (X20).]

**Figure 7:** EPCs after 7 days in culture (X20).
cells. After 7-10 days, the cells demonstrated a spindle shape morphology (Figure 7), indicative of early outgrowth EPC’s and consistent with previous reports.\textsuperscript{79}

Immunohistochemistry staining demonstrated that the cultured EPCs stained positive for CD34, CD133, Fetal liver kinase (Flk1), and von Willebrand Factor (vWF), indicating an appropriate cell population for EPC therapy (Figure 8).

**Figure 8:** EPCs stain positive for CD34, CD133, Flk1, and vWF (nuclei counterstained with DAPI).
**MSCs**: MSC culture revealed spindle/fibroblastic shaped cells adherent to plastic after 5-6 days in culture. By the third passage a relatively homogeneous population of MSCs was apparent (Figure 9).

Figure 9: Relatively Homogenous MSC population after passage 3 (X20).

Immunohistochemistry staining demonstrated that passage 3 MSCs stained positive for CD29, CD90, CD44, and CD105, indicating an appropriate cell population for MSC therapy (Figure 10). These staining results demonstrated that our MSC cell population did lack homogeneity for some markers (particularly CD29 and CD105).
Figure 10: MSCs stain positive for CD29, CD90, CD44, and CD105 (nuclei counterstained with DAPI).
4.2 Plain Radiograph Assessment of Bone Defect Healing

AP radiographs were conducted every 2 weeks. In the EPC group, the majority of specimens (86%) demonstrated radiographic evidence of early callus formation at 2 weeks post-

Figure 11: AP Radiographs comparing progressive healing of a bone defect in a rat femur between control defects, MSC treated defects, and EPC treated defects. The same rat is shown in each series.
operatively, and all specimens demonstrated callus formation by 4 weeks. By 6 weeks, 4/14 (29%) specimens in the EPC group were classified as completely healed and the remaining 8/14 (71%) were classified as partially healed. By 10 weeks post-operatively all 14 specimens in the EPC group were classified as completely healed (Figure 11). In contrast, the MSC group demonstrated minimal evidence of bone formation at any time point after surgery. In the final 10 week radiographs, 1/14 (7%) specimens was classified as partially healed and the remaining 13/14 (93%) were classified as not healed. The control group also demonstrated minimal evidence of bone healing throughout the study period. In the final 10 week radiographs, 1/14 (7%) specimens was classified as partially healed and the remaining 13/14 (93%) were classified as not healed (Table 2).
### Treatment Group

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Ten Week Radiograph Results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Not Healed</td>
</tr>
<tr>
<td></td>
<td>Number of Specimens (%)</td>
</tr>
<tr>
<td>Control (n=14)</td>
<td>13 (93%)</td>
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<td>EPC (n=14)</td>
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</tr>
<tr>
<td>MSC (n=14)</td>
<td>13 (93%)</td>
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</table>

Table 2: Radiographic results at 10 weeks post surgery in the 3 different groups.

### 4.3 MicroCT Assessment of Bone Defect Healing

Qualitative assessment of the 3D reconstruction images of microCT analysis of the 10 week specimens from each group demonstrated consolidated bone formation at the bone defect site in the EPC group versus minimal bone formation at the defect site in both the MSC and control groups (Figure 12).
Quantitative assessment of individual microCT parameters is shown in Table 3. The EPC group showed significantly improved bone formation in all parameters tested relative to both the MSC and control groups. There were no significant differences between the MSC and control groups in any of the parameters tested.
<table>
<thead>
<tr>
<th>MicroCT Parameter</th>
<th>Experimental Group</th>
<th>P value (EPC vs MSC)</th>
<th>P value (EPC vs Control)</th>
<th>P value (MSC vs Control)</th>
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</thead>
<tbody>
<tr>
<td>Mean BV (mm³)</td>
<td>EPC 36.8197, MSC 23.3986, Control 20.5407</td>
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<td>.006</td>
<td>.826</td>
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<td>SE</td>
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<td>Mean BV/TV (%)</td>
<td>EPC 0.2189, MSC 0.1030, Control 0.1076</td>
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<td>&lt;.001</td>
<td>.950</td>
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<tr>
<td>SE</td>
<td>EPC 0.01428, MSC 0.00559, Control 0.01139</td>
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<tr>
<td>Mean Conn D (1/mm³)</td>
<td>EPC 96.7332, MSC 38.6613, Control 34.6613</td>
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<td>Mean BS (mm²)</td>
<td>EPC 568.1377, MSC 321.0622, Control 291.1078</td>
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<td>.014</td>
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<td>Mean TbN (1/mm)</td>
<td>EPC 1.5209, MSC 0.6704, Control 0.7852</td>
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<td>Mean TbS (mm)</td>
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<td>EPC 0.05075, MSC 0.11079, Control 0.21147</td>
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</tbody>
</table>

**Table 3:** Mean quantitative microCT values, standard errors (SE), and p values for each parameter tested. Significant p values are highlighted in red.
4.4 Biomechanical Testing

Biomechanical testing showed the EPC treated defects had significantly higher torsional strength and stiffness than both the MSC and control defects (Figures 13 and 14). The torsional strength of EPC treated defects was 37% of that of the contralateral intact femurs and the torsional stiffness was 43% of that of the contralateral intact femurs.

Figure 13: Maximum torque values for each experimental group (* indicates p<.05)
4.5 Histology (Bone Healing)

Qualitative analysis of the decalcified 10 week histology specimens from the EPC treated defects revealed abundant callus formation and ossification across the entire gap. The bone
defect site was filled with newly formed trabecular bone. In contrast, slides from the control groups showed patchy filling of the defect with newly ossified areas and predominantly fibrotic tissue (Figure 15).

4.6 Laser Doppler Perfusion Imaging of Soft Tissue Blood Flow

The LDPI data obtained from all three groups is presented in Figure 16. All 3 groups demonstrated an increase in soft tissue blood flow at the fracture site relative to the non-operated limb at 1 hour, 1 day, and 3 days post-operatively. There were no significant differences in the blood flow measurements obtained from the 3 groups at this time point. However, beginning at 1 week post-operatively and continuing out to 3 weeks, the EPC group
showed a progressive increase in soft tissue blood flow at the fracture site that was not observed in the MSC or control groups. The EPC group demonstrated significant differences from both the MSC and control groups at these time points (Figure 16). There were no significant differences observed in soft tissue blood flow between the MSC and control groups at any time point. Representative laser Doppler images from the EPC and MSC groups at 2 weeks post-operatively, showing increased fracture site blood flow in the EPC group are shown in Figure 17.
Figure 16: Mean soft tissue blood flow for EPC, MSC, and control groups at 1 hour, 1 day, 3 days, 1 week, 2 weeks, and 3 weeks post-operatively expressed as a ratio of fracture site blood flow relative to the corresponding region on the non-operated limb. (* indicates significant difference between EPC and MSC/control groups).
4.7 Laser Doppler Probe Assessment of Bone Blood Flow

The LDF data obtained from all 3 groups is displayed in Figure 18.

The LDF data demonstrates that there was a significant decrease in bone blood flow immediately following creation of the osteotomy in all 3 groups, with no significant differences.
Figure 18: Mean values of LDF data from all 3 anatomic sites where bone blood flow was measured. (* indicates significant difference between the EPC group and MSC/Control Group).
between the groups observed. This phenomenon was observed both proximal and distal to the bone defect site (fracture gap). It was not possible to measure bone blood flow at the bone defect site immediately post-osteotomy as this piece of bone was removed following the osteotomy, and was therefore recorded as zero. The 3 week and 10 week data for all 3 anatomical sites demonstrates significantly increased bone blood flow in the EPC group versus both the MSC and control groups, with the exception of the 10 week group at the fracture gap. At this time point the EPC data demonstrated wide variation and the difference between the EPC group and the MSC group was significant, however the difference between the EPC group and the control group was not statistically significant. There were no significant differences in mean bone blood flow at any time point or anatomical location between the MSC and control groups.

4.8 Histology (Angiogenesis)

Qualitative analysis of the 3 week histology specimens from the EPC treated defects revealed numerous blood vessels apparent and evidence of new bone formation at the defect site. In
contrast, slides from the control groups demonstrated very few blood vessels and rare evidence of bone formation (Figure 19).

**Figure 19:** Histology slides from the bone defect site in EPC and control groups. The EPC slide demonstrates more blood vessels (black arrows) and newly formed bone.
Chapter 5: Discussion

The central aim of the current study was to compare the effects of two types of stem/progenitor cells (EPCs and MSCs) on the healing of critical sized bone defects in an animal model. Our primary hypothesis was that EPCs would demonstrate superior bone healing and angiogenesis relative to both MSCs and control treated defects.

The first aim of the current study was to compare the effects of EPC therapy, MSC therapy, and control treatment on healing of a bone defect. The radiographic results demonstrated that control treated defects reliably result in nonunion of the bone defect confirming that our model in fact represents a ‘critical sized bone defect’, where the defect fails to heal in the absence of treatment. This finding is consistent with our previous studies. In contrast EPC treated defects demonstrated callus formation as early as 2 weeks with complete healing of all defects by 10 weeks. These results demonstrate that EPC therapy consistently regenerates bone in the setting of a critical sized defect. We were somewhat surprised by the finding that MSC therapy had no influence on the radiographic outcome in the MSC treated defects. The radiographic results demonstrated no benefit of MSC therapy over control treatment alone, a finding that was confirmed by microCT and biomechanical testing. We had expected superior bone healing
in the EPC group versus the MSC group, but had anticipated that MSC therapy would demonstrate some improvement over controls. This finding and possible explanations are discussed later in this section.

The microCT results provide objective confirmation of the radiographic results, demonstrating significant differences in all parameters of bone formation for the EPC group over both the MSC and control groups. The microCT results also confirm that there was no difference between the MSC group and controls with regard to bone formation, once again suggesting a lack of benefit from MSC therapy in this bone defect model. Furthermore, the microCT results demonstrated superior 3D structure of the EPC regenerated bone based on the significant differences observed in the parameters tested.

The results of the biomechanical testing demonstrated that the EPC treated defects had superior torsional strength and stiffness relative to both MSCs and controls. The low mean values of torsional strength and stiffness observed in the MSC treated and control defects are consistent with the fact that these animals failed to heal their defect and presented with a mechanically unstable nonunion. Once again, no effect on biomechanical strength was observed with MSC treatment over controls. When compared to intact contralateral femurs, EPC treated defects demonstrated significantly lower torsional strength and stiffness,
demonstrating less than 50% of the strength and stiffness of intact specimens. There are several possible explanations to this. First, the biomechanical testing required plate removal from the EPC treated specimens and left the empty screw holes as a potential source of failure during loading. Some of the EPC specimens did demonstrate failure through the screw holes during loading. In addition, it is possible that further time, or removal of the plate with a subsequent period of bone healing, would have been required for the bone to remodel and attain its pre-operative functional strength.

Taken together, the radiographic, microCT, and biomechanical results demonstrate a comprehensive assessment of bone healing that shows clear favor to EPC treatment over MSC treatment or controls in the healing of this bone defect model, confirming the primary hypothesis of this study.

The second aim of the current study was to assess the effect of EPC therapy on angiogenesis at the fracture site relative to MSC therapy and controls in a bone defect model. In order to do this we elected to use laser Doppler assessment of both soft tissue and bone blood flow as both of these are critical to the progression of bone healing. In addition, laser Doppler allowed us a real-time assessment of physiologic blood flow in both of these areas. This was further supplemented with histological comparison of blood vessel formation at the bone defect site.
The LDPI results demonstrated an early increase in blood flow in the soft tissues around the fracture site that was consistent in all three groups. This was attributed to an initial hyperemic/inflammatory response in reaction to the trauma of surgery and has been reported before. After 3 days a divergence was observed between the EPC group and the MSC and control groups. Both MSC and control groups showed a progressive decline in blood flow with a return baseline blood flow levels at 3 weeks. There were no significant differences observed between the MSC and control groups, suggesting that MSC therapy has no effect on soft tissue blood flow at the fracture site. In contrast, EPC treated defects showed a progressive increase in soft tissue blood flow at the fracture site that peaked at 2 weeks after surgery. Soft tissue blood flow was significantly increased over both MSC and control treated defects at 1 week, 2 weeks, and 3 weeks post-operatively. This timeline for EPC mediated increases in soft tissue blood flow is consistent with the timeline for angiogenesis which has been reported to occur during the first 1-2 weeks at the fracture site, with the first week being the most critical for fracture healing. These results suggest that the increased soft tissue blood flow observed with EPC treatment may be attributable to EPC mediated angiogenesis and is consistent with reports in the literature of EPC mediated angiogenesis in other ischemic conditions.
The LDF results demonstrated a significant decrease in bone blood flow immediately following osteotomy of the bone. This occurred both proximal and distal to the bone defect site and was consistent across all 3 experimental groups. This may be attributable to either physical damage to both the periosteal and endosteal bloody supplies during creation of the osteotomy or to an immediate vasoconstrictive reaction in response to the trauma. Both the MSC and control groups demonstrated decreased blood flow relative to pre-osteotomy values at all 3 anatomical locations at both 3 and 10 weeks following surgery, indicating ongoing impairment of blood flow. This was quite likely an important contributing factor to the development of nonunion at the bone defect site. Once again, no differences were observed between the MSC and control groups at any location or time point, indicating no effect of MSC therapy on bone blood flow.

In contrast, EPC treated defects demonstrated significant increases in bone blood flow at all anatomical locations both at 3 and 10 weeks. The increases in bone blood flow observed at 10 weeks despite a healed fracture are consistent with reports in humans of increased bone scan activity in healing fractures up to a year following injury. These physiological measures of blood flow activity at both the level of the bone and soft tissues were supplemented by the histology results which demonstrated an increased number of blood vessels visible at the bone defect site at 3 weeks in EPC treated defects versus control
treated defects. Taken together these results suggest that EPC treatment has a significantly positive effect on blood flow and new vessel formation at the desired site of bone regeneration that is superior to MSCs, confirming the second hypothesis of the current study.

Several issues warrant further discussion. The first is the lack of any positive effect of MSC therapy on healing of the bone defect or angiogenesis in our study. As mentioned earlier, we had hypothesized a superior effect of EPC therapy over MSC therapy, but had anticipated that MSCs would have demonstrated some positive effect on bone healing over controls. There are several potential explanations to this. First, it is possible that MSCs are ineffective unless combined with an osteoconductive scaffold. Previous pre-clinical studies have demonstrated that MSCs can heal bone defects in combination with an osteoconductive scaffold such as a ceramic cylinder.\textsuperscript{30, 34, 35} Kadiyala et al were able to heal 8mm segmental femoral bone defects in a rat model with MSC-loaded porous cylinders of hydroxyapatite/tricalcium phosphate.\textsuperscript{33} Similarly, Bruder et al demonstrated healing of segmental defects in the femora of adult female dogs using MSC-loaded porous ceramic cylinders consisting of hydroxyapatite (65 %) and $\beta$-tricalcium phosphate ceramic (35 %).\textsuperscript{34} We elected to use gelfoam (a collagen sponge) as our scaffold because it is inexpensive, easy to use, allows ongoing radiographic assessment as it is radiolucent (in contrast to ceramic scaffolds which are radiopaque), and the use of a collagen
scaffold for cell-based therapy is well described in the literature.\textsuperscript{49, 67, 72} In addition, we felt that the use of gelfoam would allow us to directly compare the osteogenic/angiogenic potential of the cells themselves. However, it is possible that we may have observed different results if we had compared EPCs and MSCs on an osteoconductive scaffold. In addition, previous investigators have demonstrated that the stiffness of the microenvironment to which MSCs are exposed can influence their differentiation down a specific pathway independent of other factors. Engler et al demonstrated that exposure of MSCs to a rigid matrix that mimics the extracellular matrix of bone leads to osteogenic differentiation whereas a less rigid matrices lead to myogenic or neurogenic differentiation.\textsuperscript{84} It is possible that our gelfoam scaffold wasn’t rigid enough to promote osteogenic differentiation of the MSCs, further supporting the need for future experimentation with different scaffolds. A further possible explanation is that MSCs are ineffective unless combined with osteoinductive/angiogenic proteins. Of note is the fact that gene therapy studies which have demonstrated positive results with genetically modified MSCs have typically used non-genetically modified MSCs as a positive control and reported that MSC therapy alone results in nonunion of the bone defects.\textsuperscript{42, 43, 49} Again, this is consistent with our initial hypothesis that MSCs are ineffective as a cell-based therapy for bone regeneration on their own. Finally, our staining results demonstrated that our MSC population did lack homogeneity for certain MSC markers (CD29 and CD105). This raises questions regarding the
purity of our MSC population, and could have affected our results. However, it is important to note that we followed a standardized protocol for MSC isolation that has been previously described and validated in the literature.\textsuperscript{75}

Second, we observed that EPC therapy had a positive effect on both bone regeneration and angiogenesis in our model, confirming our initial hypothesis. We observed an association between blood flow, vessel formation, and bone regeneration. However, on the basis of the experimental evidence presented here we are unable to conclude that there is a causal relationship between the bone regeneration observed and angiogenesis. In fact the mechanism by which EPCs augment fracture healing remains poorly defined. It is unclear whether EPCs improve bone regeneration by creating new blood vessels, differentiating into bone-forming cells, or by secreting trophic factors such as VEGF and BMP which stimulate both bone formation and angiogenesis. It is possible, and seems likely, that it is a combination of these factors. Further investigation is required to clarify these issues. In addition, the role of endogenous EPCs in normal fracture healing is poorly defined as the investigation of this cell type in orthopaedics is relatively novel. The fact that EPCs play a pivotal role in angiogenesis in other ischemic conditions, suggests that they likely play an important role in the early stages of
fracture healing as well. Further research into the function of EPCs in the normal fracture healing cascade is required.

Finally, fracture healing is a complicated molecular and multicellular process and trying to emulate that with a single cell strategy may be overly simplistic. Given the multicellular nature of fracture repair, the best strategy may involve a combination of EPCs and MSCs, as opposed to either cell type on its own. The purpose of this study was to take the initial step and compare the two cell types as a single-cell strategy to determine which type was superior in our model. Certainly, the prospect of combining the two cell types is an enticing one, which warrants further investigation.

Our results represent a significant departure from previous literature on cell based strategies for bone regeneration which have focused primarily on MSCs due to their documented ability to differentiate into bone forming cells. Our strategy of using EPCs, a progenitor cell with known capacity to differentiate both into bone forming cells and blood vessels, proved to be superior to MSC treatment in our model. This strategy addresses both the impaired vascularity at the site of bone regeneration and the need for bone forming cells. Furthermore, it is able to accomplish this without the need for gene therapy. Our findings represent a significant stride
forward in the tissue engineering of bone and potentially bring us one step closer to finding a
superior treatment for bone defects and nonunions in orthopaedic patients.
Chapter 6: Conclusions

The primary aim of the current study was to compare the effects of two types of stem/progenitor cells (EPCs and MSCs) on the healing of critical sized bone defects in a rat model. The results of the current study clearly demonstrate that EPC therapy results in superior bone healing relative to both MSCs and controls, confirming the central hypothesis of the study.

Our secondary aim was to compare the effects of these two cell types on local angiogenesis at the site of desired bone regeneration. EPC therapy clearly demonstrated superior blood flow and blood vessel formation at the bone defect site relative to both MSC therapy and control treatment, confirming our second hypothesis. Taken together these results suggest that EPC therapy results in superior osteogenesis and angiogenesis relative to MSC therapy. EPC therapy represents a promising potential treatment for the management of bone defects and nonunions in orthopaedic patients.
Chapter 7: Future Directions

While the results demonstrated with EPC therapy in the current study are impressive, it is important to remember that the investigation of EPC therapy for fracture healing is in its infancy compared to the decades of research that have already been conducted on MSC therapy. To our knowledge, the first investigation of EPC therapy for bone regeneration was published in 2006\textsuperscript{70}, and only a handful of other studies have been published on the topic since then. A number of critical issues regarding EPC therapy for the regeneration of bone need to be clarified with further research. First, the mechanism by which EPCs stimulate bone healing and angiogenesis at the fracture site needs to be elucidated. This will require cell labeling studies, innovative imaging strategies, and quantification of growth factor expression at EPC treated bone defects. Second, it is critical that the role of endogenous EPCs in the native fracture healing process is further elucidated. This will involve investigation of the role of EPCs in the early stages of the fracture healing process and may require novel strategies (such as a fracture model in a mouse whose native bone marrow is lethally radiated, and subsequently transplanted with fluorescently labeled cells).
As mentioned earlier, the prospect of combined EPC and MSC therapy for the regeneration of bone is an enticing one and represents the next logical step from the investigation presented here. Such investigation should involve experimentation with EPCs and MSCs on different types of scaffolding as well, including osteoconductive scaffolds. In continuing my research I would begin with this step, and explore different combinations of EPCs and MSCs both in an in vivo model of bone defect healing and an in vitro co-culture model. In vivo experiments would involve comparing EPCs, MSCs, and EPC/MSC combinations in different ratios (ie 1:1, 2:1, and 1:2) in an animal model of a bone defect similar to the one described here. I would also like to investigate the influence of different scaffolds. As mentioned in the discussion, several authors have reported positive results with the use of MSC-loaded ceramic scaffolds and, as such, I think that a comparison of EPCs and MSCs on such a scaffold warrants further investigation. In vitro experiments would include a co-culture system of osteoblasts and EPCs, MSCs, or EPCs/MSCs to examine the influence of these different cell types on osteoblast proliferation and mineralization in culture.

Finally, while the strategy of culture expansion and subsequent transplantation of EPCs proved to be an effective one here, such a strategy is cumbersome, time-consuming, and expensive in the clinical setting. In our mind the ideal bone graft substitute would be available as an ‘off-
the-shelf’ type of product. We envision EPC investigation in fracture healing potentially leading to such a product in one of two ways. The first involves the use of allogeneic EPCs, whereby EPCs harvested and cultured from healthy donors is available ‘off-the-shelf’ in much the same manner in which blood transfusions currently are. In support of this possibility is some early evidence in the cardiovascular literature that EPCs are immune privileged and may not elicit an immunogenic response if transplanted among different individuals.\(^85, 86\) The second involves the investigation of growth factor delivery which may potentially mobilize and stimulate the patient’s endogenous EPC population or augment their native EPC response. Several such growth factors have already been identified in the cardiovascular literature (VEGF, angiopoeitin-1 [Ang-1], stromal cell- derived growth factor-1 [SDF-1], granulocyte-macrophage colony-stimulating factor [GM-CSF], FGF, and erythropoietin)\(^87\) and represent promising potential therapeutic candidates for stimulating fracture healing.

These represent but a few of the many potential avenues for the progression of the research presented here. This myriad of options highlights the fact that we are currently in an exciting time for tissue engineering research in orthopaedics and musculoskeletal medicine.
References


