CHARACTERIZATION AND ASSESSMENT OF LUNG AND BONE MARROW DERIVED ENDOTHELIAL CELLS AND THEIR BONE REGENERATIVE POTENTIAL

Conner R. Valuch

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STATEMENT OF COMMITTEE APPROVAL

Dr. Jiliang Li, Chair

Department of Biology

Dr. Melissa Kacena

IU School of Medicine, Department of Orthopedic Surgery

Dr. James Marrs

Department of Biology

Approved by:

Dr. Theodore Cummins

To family, friends, and my pooch, Wallace

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ABSTRACT

Fracture repair is costly and difficult to treat. One of the main causations of nonunion is a lack of essential blood supply. The needed blood is supplied by the growth of new blood vessels, a process known as angiogenesis, that invade the damaged tissue early in the healing process. We proposed using bone tissue engineering as an effective therapy. This therapy uses stem cells to aid in tissue regeneration. Endothelial progenitor cells (EPCs) were selected due to their ability to form tube-like networks in vitro. EPCs were isolated from murine bone marrow and lung tissue. We tested EPC's tube forming, proliferative, and wound migration ability in vitro. To test their ability in vivo we created a femoral fracture in young and old mice. EPCs were seeded to the fracture site upon a collagen scaffold. The in vitro studies displayed that the bone marrow and lung derived endothelial cells presented EPC traits. In the mouse fracture model bone marrow endothelial cells did not significantly improve the healing process. In the future we want to improve our cell extraction and purification method, as well as test a new stem cell delivery biomaterial. We also want to select and use a growth factor (GF) that can help to promote bone regeneration in tandem with the EPCs.

CHAPTER 1. INTRODUCTION

Bone fractures are a common and costly medical trauma. It is the highest frequency hospitalization-trauma in the United States. [1] This puts a large cost strain on an already overburdened healthcare system, as well as emotional, physical, and economical strain on the individuals suffering from said trauma.[2] Despite improved surgical techniques, about 10% of fractures result in delayed- and non-unions. [2-5] There are different definitions for non-union. According to the U.S. Food and Drug Administration (FDA) a non-union is a fracture that has failed to heal in at least nine months and has not shown any progression for three months. [2] Non-union treatment can require multiple surgeries, which results in prolonged treatment that affects the patients mental and physical health. [2, 6] Immobilization caused by non-union, in older patients, can lead to atrophy and an increase in disease susceptibility ultimately resulting in death. [7] Improper osseous healing can result in difficult impairments, such as disfigurement and even amputation. [8] The aging population in the US, ages 65 and up, is expected to compromise 17% of the total population by 2030. [9] Aging can lead to decreased bone quality and delayed fracture repair, which can be attributed to a myriad of physiological changes. [10] Patients with osteoporosis are especially susceptible. [10] Fractures that occur to patients with osteoporosis significantly increase the disability and mortality rate in patients. [11]

There are a multitude of problems that can lead to impaired fracture healing. Soft-tissue damage, vascularization obstruction, infection, tumors, and lack of mechanostability are among the chief issues. [4] A poor blood supply appears to be the most common reason for non-unions.[5, 12] Bacterial infections are another common cause, but these are mostly associated with open fractures obtained from high-energy trauma. [2] Our understanding of bone repair and the surgical treatments have improved, however, the rate of non-union remains significant. [2, 4] The current therapies have their separate risks and do not reduce the rate of impaired healing. A well-rounded option that bridges both the biological and mechanical properties necessary for proper healing is on the horizon. This promising therapy is bone tissue engineering (BTE). There are four pillars of BTE: structural stability, cell signaling, stem cell support, and mechanical stimuli. [13, 14] These foundations provide a balanced regenerative structure that should enhance healing the process and reduce the rate of delayed- and non-union.

Researchers have a much better understanding of how bone healing works and what issues can lead to bone healing impairment. A lack of poor blood supply is one of the most common causes, though other risk factors such as age and diabetes can contribute to impaired healing too. BTE is a promising new therapy option that embraces both the biological and mechanical aspects of fracture healing. We propose using EPCs to help induce blood vessel formation within the fracture callus. Our research looked at two separate EPC populations, one derived from bone marrow and the other from lung tissue. Through a set of in vitro experiments, we tested their proliferative, vessel-forming, and collective migratory ability, as well as their genetic profile regarding vasculature formation. After in vitro data was collected, we performed an *in vivo* study using a murine fracture model that tested whether implanted EPCs improved bone healing.

1.1 Fracture Repair Process

Bone is one of the few tissues that can heal without leaving a fibrous scar. [2] Most fractures heal naturally, though if the bones are not set back in their correct anatomical position deformities can remain. [15] There are two main categories of fractures to consider. Bone fractures can either be closed or open fractures, though there are different types of breaks within these divisions. Open fractures result in breakage of the skin and are typically more severe because they expose the healing site to environmental elements. [2] Closed fractures are not exposed to the outside environment but have their own issues. The severity of the break is often what causes healing complications. [8] In the case of an open fracture, for instance, a higher rate of low-grade infection occurs. [2] Increased damage to epithelial and muscle tissue can decrease stability and blood flow to the injured area, compounding the stress on the fracture. [5]

There are two forms of bone healing: primary and secondary. [3] Secondary fracture healing is the most common form of bone repair, which consists of both endochondral and intramembranous ossification. [16] In secondary healing, instability at the fracture site induces a callus that provides stability and becomes the template for ossification. [3, 5] In the primary model, the fracture site is sufficiently compressed and stable, which prevents a callus from needing to be formed. [3] Endochondral ossification is associated with long bone healing. [12] Intramembranous ossification, will nominally occur when there is better blood supply, and MSCs differentiate into osteoblasts

(OBs) that then promote bone formation. [5, 12] Bone healing is not only dependent on biological criteria being met, but also on mechanical conditions. Stability is a key factor in bone formation.

There are four main phases of secondary bone healing, (1) inflammation, (2) soft callus formation, (3) hard callus formation, and (4) bone remodeling. [12] Blood vessels are severed from the trauma. As a result, blood hemorrhages from the vessels into the surrounding tissue and begins to clot, forming a hematoma. [3, 9, 12, 16] The hematoma is the initial template for the callus. The inflammation phase occurs within the hematoma. [3] This inflammatory period involves a brief, but important, increase in proinflammatories that are essential for proper tissue regeneration. [3, 9, 16] Of those proinflammatory cytokines, IL-1, -6, -11, and -18, as well as Tumor Necrosis Factor alpha (TNFα) are released into the surrounding tissue. [16] These cytokines recruit inflammatory cells, such as neutrophils, to the wound area to phagocytize debris and microbes. IL-1 and -6, in particular, play important roles in promoting vascularization, increasing vascular endothelial growth factor (VEGF), and inducing cartilage formation.[16] The hematoma is so important that in cases where the hematoma is removed, the healing process is significantly impaired while the inverse occurs when hematomas are transplanted into fracture sites. [12]

Phagocytic cells begin to clear away dead cells, debris, and microbes. [16] New blood vessels provide oxygen and nutrients to the metabolically active callus and provide a route for inflammatory, cartilage, and bone precursor cells to arrive. [17] In order for bone to regenerate, MSCs must be recruited to the callus to differentiate into osteogenic cells. [16] These cells are recruited from multiple regions, including bone marrow, surrounding soft tissues, cortex, and periosteum. [16] MSCs differentiate into OBs and chondrocytes, among other cell types. [5] The hematoma will eventually be replaced by a soft cartilage callus, directed by MSC differentiation into chondrocytes via chondrogenesis. [2, 16] The chondrocytes secrete an avascular cartilage matrix while new bone formation occurs at the periphery. [9] Chondrocytes will eventually undergo apoptosis leading to enzymatic degradation of the cartilaginous matrix in order for osseous tissue to replace it. [2] Upon formation of the collagen matrix, blood vessels permeate the area and initiate the next phase of bone repair, bone mineralization. [16] OBs derived from MSC differentiation, and present in nearby tissue, deposit calcium phosphate in the extra cellular matrix, forming osseous tissue. [2] This resulting osseous matrix is known as a hard callus. This leads to the last stage of bone repair, which is bone remodeling. Bone remodeling reshapes the bone back

into its previous structure, reducing the callus and replacing the spongy bone with compact, cortical bone.[16] This process takes several months and can even take years. [3, 16]

1.2 Blood Vessel Formation

Blood vessels play an essential role in bone healing. A lack of vascular perfusion at the fracture site compromises the oxygen and nutrient supply as well as the disposal of wastes and toxins, leading to cell death, poor integration, and graft failure. [18] Furthermore, studies have suggested that blood vessel formation and osteogenesis are coupled, which is indicative of observed molecular cross talk between osteogenic and endothelial cells. [12, 19] Previous reports have shown that the rate of non- and delayed-union can occur as high a rate as 46% in fracture patients with accompanying vascular injuries. [18] Intentionally disrupting vasculature perfusion with anti-angiogenic agents inhibits proper bone formation during fracture healing. [20, 21] In older patients, the bone tissue has decreased vascularization, which may lead to decreased vessel formation upon fracture. [9] An adequate blood supply is something that must be addressed in proper bone healing. There are two different types of vasculature formation. The first type, angiogenesis, is the result of new vessels forming from existing vasculature. [17, 19, 22] There are two subtypes, sprouting and intussusceptive angiogenesis. [23] The prior refers to new tubes sprouting from existing tubes and the latter occurs when a single vessel reforms into two separate vessels. [23] The second type of blood vessel growth is referred to as vasculogenesis. Vasculogenesis is the generation of completely new vessels from EPCs. [17, 18]

Blood vessels are composed of lining cells known as endothelial cells (ECs). These cells are derived from hemangioblasts, which are multipotent SCs. [19] Hemangioblasts differentiate into EPCs or hematopoietic stem cells (HSCs). EPCs can go on to become ECs, whereas HSCs are responsible for differentiation of most blood cells varieties and several immune cells. [19] Initiation of angiogenesis and vasculogenesis both depend on GFs and cytokines. VEGF, fibroblast growth factor (FGF), tumor necrosis factor-alpha (TNF-α), transforming growth factor-beta (TGF-β), and the angiopoietins (ANGs) are a few of the most common angiogenic GFs. [24] These molecular drivers will either recruit EPCs to an area for vasculogenesis or will bind and activate ECs within blood vessels, causing them to sprout or initiate intussusceptive splitting. [25]

Of the two forms of angiogenesis, sprouting angiogenesis is much more prevalent and well understood. Sprouting angiogenesis is typically initiated due to low levels of oxygen supply, hypoxia, for parenchymal cells (function cell of an organ - nerves, myocytes, myocardial cells, etc.). [26] Mechanical sensors initiate signal cascades which causes GFs and cytokines to rendezvous in regions where blood vessel sprouts will develop. One endothelial cell will be activated by GFs and become a tip cell that acts as an apical bud, a terminal point of vessel growth. [25] The growing sprout is referred to as a stalk. Cells behind the tip cell are known as stalk cells. These cells are responsible for proliferation, forming a lumen, and thus elongation of the stalk. [25] During quiescence and stabilization of new vessels, tip cells repurpose into phalanx cells, which do not proliferate, but help to stabilize the blood vessel.[23]

1.3 Current Fracture Treatments

Bones recovering from fracture need four basic conditions to be met for proper healing: stability, blood supply, aseptic environment, and appropriate soft tissue management. [8] Although the timeframe for healing varies based on the severity of the break, adequate healing usually occurs around 6-8 weeks. [13] When the bone takes significantly longer to heal, it is known as a delayed union, and when the bone fails to heal it is called a non-union. [6, 8] There are multiple causes of nonunion including mechanical instability, insufficient vascularization, periosteal disruption, infection, and poor soft tissue coverage. [6] Healing can also be affected by patient specific problems, such as: age, smoking status, alcohol intake, genetic predisposition, severe anemia, and diabetes. [13, 27] There are both nonsurgical and surgical options for treating fracture healing impairment. A medical option of note is the use of pulsed electromagnetic waves. A bone stimulator creates electromagnetic waves, which stimulate osteogenic markers that upregulate proliferation and differentiation, thus increasing osteogenesis. [7]

In the case of non-unions, the lack of stability and the severity of damage often call for a surgical treatment. [3, 4] In many cases some type of graft material or metal fixative provides stability and accounts for the lost bone material.[4] Despite the improvement in these surgeries, side effects, such as bone loss, infection, and soft-tissue damage still remain. [4] The other major surgical method is bone transplantation, more commonly known as bone grafts. [15] Bone grafts are the second most used tissue transplanted in the United States. [28] There are two main types of grafts,

allografts and autografts. Allografts are harvested from cadavers and autografts are derived directly from the patient. [15, 28, 29] Autografts are considered the gold standard for bone trauma because they confer the lowest risk of immunological infection and have strong osteoconductive, osteoinductive, and osteogenic properties. [28, 29] The downside of autografts is that the surgical invasiveness of harvesting bone tissue is risky and that there may not be enough bone tissue available. [28] Allografts are advantageous because they bypass the invasiveness and lack of abundant tissue, although they are more likely to have reduced osteogenicity and osteoinductivity.[4, 15, 29] Bone grafts have a multitude of side effects: infection, bleeding, blood clot, nerve damage, complications from anesthesia, and infection from the donated bone. [30]

Bone morphogenetic proteins are another therapeutic for non-union. [29] BMP2 is currently the only FDA approved clinical drug used for bone formation and bone graft substitution. [31] BMP-2 SMAD signaling induces, among many other factors, Runx2 in OBs to induce osteogenesis. [31, 32] The induction of vasculogenesis is another method that BMP-2 appears to increase bone formation through.[10] Despite these benefits of BMP-2, a growing wave of negative side effects is pushing researchers to finding better options.

[31] Inflammation, ectopic bone formation, Increased osteoclast activity, wound complications, and increases risk of cancer. [10, 31, 33] An abnormal upregulation of BMP2 in different organ specific tumors has been observed and BMP-2 has been associated with tumor proliferation and invasion. [31]

1.4 Components of Bone Tissue Engineering

BTE has four main components: (1) SCs, (2) scaffolds, (3) GFs, and (4) mechanical stimulation. [13] SCs are cells that have the potential to differentiate into other cell types. This ability is known as potency. Some of the SCs implicated in bone and endothelial cell differentiation are MSCs, EPCs, embryonic SCs, Induced SCs, and perinatal SCs. [13] Biomaterials include synthetic and naturally derived scaffolds, which deliver SCs and GFs to the fracture site, while providing a niche for bone healing. GFs induce molecular pathways that affect bone repair, which has already been discussed at length. Thrombopoietin (TPO), platelet derived growth factor (PDGF), VEGF are all strong candidates for increasing bone formation and angiogenesis within fractures. Finally, certain mechanical stimuli help to initiate everything, activating the separate components and promoting

the concerted effort of each to repair bone. [13] Perfusion bioreactors help improve gradient movement of nutrients and signals through the scaffold, and also seem to stimulate osteogenesis and bone mineralization via osteoclasts within the scaffold. [13]

1.4.1 Stem Cells for Therapy

Looking at the possible cells for BTE, EPCs standout as an SC with strong potential. A major issue with tissue engineering is that the scaffold needs blood to supply tissue regeneration immediately. [34] This stipulation makes EPCs an interesting candidate for BTE because they secrete angiogenic GFs, reducing the time it takes to form vasculature to permeate and supply the scaffold. [35] Additionally, EPCs can be isolated in a non-invasive manner from adult peripheral blood and umbilical cord blood. [35, 36] They can also be acquired from human induced pluripotent SCs. [35] Due to their ability to differentiate into any endothelial cell type, and to grow and produce blood vessels *in vitro*, it may also be possible to use autologous EPCs to develop vasculature genetically specific to the host. [35] This would allow for clinicians to do things like determining the optimal drug combinations for a specific patient. [35]

Along with EPCs, other cells such as MSCs could be used in parallel to improve EPC effectiveness. [34] MSCs are multipotent SCs that can differentiate into multiple cell types, such as adipose cells, OBs, chondrocytes, and more.[13] OBs and chondrocytes both play roles in endochondral ossification. They have already been shown to be used as a ‘reserve pool’ for tissue regeneration in injured tissues. [13] MSCs are similar to EPCs in that they can be derived from multiple tissues, consequently bone marrow, a major location of EPCs. [13] Along with their ability to differentiate into important tissue regenerative cells, and their similar tissue residence, MSCs also promote EPC cell migration, vascularization, and bone repair. [34] EPCs in turn will form the vasculature, which then allows for MSCs to migrate through the scaffold better and differentiate into OBs. [21, 37]

1.4.2 Scaffolds for Engraftment

In the case of scaffolds, just as in bone grafts, the natural or synthetic material should satisfy 3 principles: better osteoconductivity, osteoinductivity, and osteogenicity.[4, 29, 37, 38] If the model is osteoconductive, that means it will provide a structural lattice that promotes bone formation and neovascularization.[29] Hydroxyapetite (HA) is one such material. [4] It is the major inorganic component of bone and has been shown to possess strong osteoconductive capabilities. [4] An osteoinductive graft would promote the growth of new bone via the release of cytokines that induce MSC differentiation into osteoprogenitor cells. [29] Finally, an osteogenic graft is one that allows osteoprogenitor cells, OBs, and osteocytes, which can directly form new bone at the fracture site. [29]

Collagen is a promising biomaterial option that could be used as a scaffold for bone tissue engineering. It is abundantly present in the form of type I collagen in bone tissue. [37, 39] Collagen hydrogels are inherently biocompatible, biodegradable, highly porous, marginally antigenic and combine well with other materials, such as hydroxyapatite or tricalcium phosphate. [21, 37, 39] There is a lot of clinical evidence that shows collagen compatibility with tissue regeneration [37] A limitation for collagen, however, is its relatively poor mechanical structure and its pension for swelling due to its high hydrophilicity. [37]

Choosing the shape of the scaffold is another important step. Blood supply is essential to successful bone tissue engineering. New vasculature needs to quickly grow through the scaffold to facilitate the healing and regeneration process. Therefore, when considering the shape of the scaffold, it needs to be permeable. It has been shown that scaffolds with a minimum pore size of 150um and mean pore size of 300um are best suited for bone tissue formation. [37] The scaffold must also be permeable to proteins, GFs, and other essential molecules that are necessary for cell function and regeneration. [37]

1.4.3 Growth Factors

Multiple GFs may be useful in improving bone healing processes. BMP-2 has already been addressed as a clinically significant promoter of osteogenesis, although several risks are involved with its use. VEGF, PDGF, and TPO are some prospective GFs that may prove effective in BTE. VEGF is one of the most important regulators of vascular development and angiogenesis. [5] VEGF plays an important role in the proliferation, migration, and activation of ECs. [5, 40] Along with VEGF's role in vasculature development, it is also involved with osteoblast maturation, ossification, and bone turnover. [5, 41] PDGFs are similar to VEGFs in structure, and induce proliferation and migration of ECs. [42, 43] It is an important GF for blood vessel development, and another popular GF for BTE. TPO induces megakaryocyte proliferation by binding to c-Mpl, its main receptor on platelets and MKs. [41] MKs are large, rare cells that produce platelets. [44] These cells may promote osteoblastogenesis. [1] Not only does TPO signaling lead to MK-initiated bone formation, but it also is thought to promote angiogenesis of ECs. [1]

1.5 Endothelial Cells in Fracture Repair and Bone Regeneration

For tissue engineering to be successful, the cells need to come from an abundant source and express tissue-specific phenotypes. [14] For these reasons, EPCs standout as a strong candidate for use. EPCs are unique among progenitor cells, in that they share the same traits as SCs. [36] These traits being, self-renewability, potency, and proliferative capacity. [36] Most progenitor cells do not share the self-renewability trait that SCs tote. [36] Their role in neovascularization, proliferation, and ability to differentiate into ECs make them a great choice for use in ischemic tissues. [14] EPCs have been shown to migrate directly to ischemic tissues and possess multiple endothelial surface markers like CD133, VEGFR2, and CD34. [45, 46] Of note, EPCs can promote vasculogenesis of tissue-engineered bones, and improve upon osteogenesis and bone reconstruction.[45]

Literature suggests that EPCs also play a direct role in osteogenesis. [21, 47] Osteogenesis is reportedly paired with angiogenesis during neo-osteogenesis.[47] More to that point, impairment of angiogenesis decreased bone regeneration in a distraction osteogenesis (DO) model. [20, 47] There is also evidence that MSCs can differentiate into EPCs and vice versa. [21, 36] MSCs are known for their differentiation into OBs, among other cell types. [21] They too are an option for

BTE, though they struggle to localize to the site, migrate into extraneous tissue, and have a low survival rate.[15] Barati et al proposed that VEGF driven MSC and ECFC differentiation causes release of osteogenic factors that diffuse to the matrix and stimulate osteogenesis. [48]

EPCs were initially discovered by Asahara et al, in 1997 as bone marrow derived, circulating EPCs isolated from peripheral blood. [49] They isolated two cell populations with anti-CD34 or anti-FLK-1 magnetic beads, and showed that in culture, these cells displayed spindle-like morphology, organized into blood-island-like clusters, and could mature into ECs. [49] In vivo, they showed that these EPCs could home to ischemic tissues and differentiated into the ECs integrated within the newly developed vasculature. [49] EPCs were thought to be derived from hemangioblasts, cells that could differentiate into both hematopoietic and endothelial lineages.[35, 36] Both hematopoietic stem cells and EPCs share common cell-surface markers and the disruption of several genes affects both hematopoietic and EC development, suggesting they share a precursor. [50] Furthermore, the EPCs harvested by Asahara et al seemingly upregulated endothelial cell markers, while downregulating hematopoietic markers in vitro, indicating the HSCs might be capable of transdifferentiating into EPCs.[51] Some literature, however, asserts that these bm-derived EPCs (BMECs) were actually various hematopoietic stem and progenitor cells with proangiogenic effects, but did not differentiate into ECs themselves.[51] This itself is controversial. Research on vasculature development in murine embryo studies have started to promote a separate mesodermal origin for hematopoiesis and vasculogenesis, ablating the idea of a hemangioblasts precursor.[35]

Since the initial Asahara paper, the potential of EPC use for regenerative medicine has led to an explosion of research into EPCs. This has led to some ambiguity surrounding EPCs with researchers defining multiple subtypes and tissue origins while using varying methods of isolation. For this reason, it can be challenging to find a consensus that leads to better directed research. EPCs are now thought to derive from multiple tissues. Some of the major origin sites for EPCs are peripheral blood, umbilical cord blood, bone marrow, and tissue-resident cells in vasculature. [21, 35, 36, 51] Heterogeneity in the vasculogenic potential of EPCs has also been observed and thus lends to a widescale of variance in engraftment potential of EPCs to host vasculature. [35] Instead of trying to explore all the different EPC groups and attempt to explain their hierarchy, this study

will consider cells from two different subtypes, BMECs and endothelial colony forming cells (ECFCs).

1.5.1 ECFCs and BMECs

The first subtype are EPCs derived from tissue walls, peripheral blood, and umbilical cord blood. [52, 53] These cells are known for their high proliferative potential and their ability to form colonies, giving them their name. [21, 50, 52] Cell surface markers found on ECFCs are shared with ECs, such as CD146, CD105, CD31, and VEGFR2. [52, 54] ECFCs retain high telomerase activity and can be replated multiple times. [50] The cells are closely linked to the endothelial lineage. They expand quickly, can be replated multiple times without losing their phenotypes, and retain strong telomerase activity, all while providing pro-angiogenic contributions.

Of particular note to this study, ECFCs proliferative ability compared between cells derived from adult peripheral blood and umbilical cord blood, indicated that cord blood ECFCs perform better. [50] Cord blood ECFCs appeared at 15-fold higher quantities per similar blood amount collected.[50] The cells also showed up in culture a week earlier and formed larger colonies.[50] Other attributes of ECFCs that make them noteworthy candidates for tissue repair is their ability to form tube-like networks *in vitro* and that they can differentiate into MSCs. [21]

ECFCs can directly incorporate into damaged vasculature or initiate vasculogenesis. [21, 52, 54-56] Introduction of ECFCs to ischemic tissue can prompt vessel growth and repair. [50] This has been shown *in vitro* and *in vivo*. [21, 56] Lung ECFCs were specifically shown to contribute to endothelial proliferation and vasculature repair in an experimental model of Acute Respiratory Distress Syndrome (ARDS). [56]

These qualities suggest a promising future for ECFC therapy for ischemic tissues. Directly related to our study, ECFCs were added to induced compound fractures in mice, along with HA/TCP scaffolds and a type I collagen sponge. [21] This in vivo study showed that fractures healed quicker, and that bones had become stronger than before the fracture.[21] Clearly this is an important step forward, although clinical studies have yet to show the same success. These cells are isolated via

CD31, also known as platelet endothelial cell adhesion molecule 1(PECAM-1). CD31 is a widely used cell marker for ECs. [54]

The second EPC subtype, BMECs, were the first EPCs discovered. Originally, EPCs were discovered by Asahara et al in 1997 and were solely derived from the bone marrow. BMECs help give rise to new blood vessels by inducing vasculogenesis and haven been shown to aide ischemic tissue repair, fracture defects included. [45, 57] Nolan et al showed that BMECs are defined by expression of VE-cadherin, VEGFR2, $CD31^{low}$, Endoglin, and Prominin I/AC133. [58] These cells differentiated into mature ECs and contributed both structurally and functionally to tumor neo-angiogenesis. [58] BMECs are thought to be circulating angiogenic cells, meaning that they move freely through bone marrow and are recruited when needed. [50] Although there remains a strong debate over what is considered an EPC, BMECs have shown promising potential to induce blood vessel formation and osteogenesis. [47] BMECs have even helped improve callus formation and healing of the osteotomy gap in a segmental defect model. [46]

Differentiating between EPCs is tough, and more work needs to be done to define the unique subsets of EPCs. EPCs often carry very similar markers to hematopoietic cells, and it can be tough to tell the two apart. Both cell types carry CD34 for example.[51] The cells are selected with a CD34 antibody, and then it is common for ECs to be sorted out using CD31, VEGFR2, VE-cadherin, FLK1, and Tie-2, whereas hematopoietic cells will have CD45 markers that are unique to their lineage. [35, 51, 58] These shared cells markers present some lineage uncertainty, but this may also suggest that hematopoietic stem cells may differentiate into EPCs to replenish needed ECs. [51] Another issue is that there is no standard isolation and culture method used, causing uncertainty to whether researchers are really working with the same cells. [58]

EPCs, for the reasons listed, show potential as a strong stem cell therapy for inducing blood vessel formation in ischemic tissues. Both subtypes considered are essential in forming new vasculature and are capable of being cultured and expanded for clinical use. Essential functions for blood vessel development: proliferation, transdifferentiation, tube formation, cell migration, and self-renewal are present in ECP populations. The main issues with EPCs seem to be in determining what is a proper EPC, and within those subtypes, which is the best for therapeutic use.

1.6 Long-Term Research Goals

Fracture nonunion is a costly problem with growing implications attached to an aging global population. Despite the current therapies for fracture healing impairment, about 10% of fractures result in nonunion. Aging patients, especially those with added risks such as Osteoporosis, face high disability rates, which can lead to atrophy or further breaks that can be life-threatening. An adequate blood supply appears to be one of the most important factors for proper fracture healing. The first goal of this study was to compare the endothelial traits of neonatal BMECs and lung derived ECFCs (LECs) in vitro. These traits: proliferation, tube formation, and collective migration, are important for vasculature formation. Relative expression of pro-angiogenic genes was also compared between the two EPC subtypes. The second, and larger, goal of this study was to test BMEC ability to promote blood vessel formation in a femur midline fracture model. We wanted to determine if neonatal progenitor cells, which have been suggested to be more proliferative and less inclined to mutate, [59-61] may be more effective at promoting fracture healing than adult progenitor cells.

1.7 Study Aims

The first aim was to test the traits of neonatal BMECs against those of neonatal LECs. Cells were isolated from their respective tissue in neonatal mice using two separate isolation methods. There are three major endothelial behaviors associated with EPCs: proliferation, tube formation, and wound migration. The first of these, proliferation, shows EPCs ability to expand/self-renew. EPCs have varying levels of proliferative ability, and their ability to rapidly expand has clinical relevance. To engineer tissue, you need a source of cells that can be expanded and sustained outside of the body. The second trait, tube formation, was tested in similar fashion. This assay is necessary to show that EPCs can form vessel-like structures in vitro, suggesting their vasculogenic potential in vivo. The two cell subtypes were seeded on Matrigel, an extracellular matrix-like substance isolated from tumors, that enables the cells to then form tube-like networks. Wound migration tests the ability of cells to migrate collectively into damaged tissue. A scratch is made across the cells, and then the cells ability to migrate into the vacated space is observed by measuring the closing space over a specific timeframe. Testing all three of these traits allowed for us to directly compare these different EPC subtypes and determine if one displayed more consistent EPC phenotypes, and

whether they would serve as a better cell for ischemic tissue repair. Along with the *in vitro* assays, real time polymerase chain reaction (RT-PCR) was performed on six pro-angiogenic genes to determine their relative gene expression.

The other aim of this study was to test the *in vivo* ability of BMECs to affect bone repair in a murine femur fracture model. The choice was made to only test BMECs, as LECs have already been shown to have a therapeutic effect on bone regeneration. Therefore, we wanted to compare BMECs ability to see which was a better option for bone regeneration. In this experiment, BMECs were tested against mice treated with saline as a control. Fractures were completed on young adult mice (3-4 months) and aged mice (24-26 months) to compare age-related consequences on healing. The cells should show their ability to migrate into the fracture area, proliferate, and promote blood vessel growth, all of which are essential for bone repair. Bones were retrieved and μCT scanning was performed to analyze the callus healing.

CHAPTER 2. METHODS & MATERIALS

2.1 Animal Models

Old C57BL/6 mice (~26 months, n=25) and young C57BL/6 mice (~3-4 months, n=28) were used for the *in vivo* fracture study. Tie2-CreER;Td-Tomato (Tie2CreERT+) (n=23) mice were used to donate tamoxifen induced BMECs. The original generation and characterization of Tie2-CreER;Td-Tomato mice was previously described. [62, 63] Animal studies were approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee.

2.2 Isolation of endothelial cells from lungs and bone marrow

EPCs were isolated from neonatal (2-6 days of age) lungs (LECs) and femur/tibia bone marrow (BMECs). Lung tissue was isolated and harvested in a sterile environment, finely minced into small pieces, and then digested with 225 U/ml collagenase type 2 solution (Worthington Biochemical Corporation, Lakewood, NJ) for 1h at 37°C and 5% CO_2. Simultaneously, Dynabeads^{TM} Biotin Binder (Thermo Fisher Scientific, Waltham, MA) were conjugated with biotin rat anti-mouse CD31 antibody (BD Pharmingen™, San Jose, CA). Beads were washed three times with 0.1%BSA/PBS and then 10µg of CD31 antibody was mixed with the beads and then incubated while shaking at room temperature for 1 hr. The minced tissue was filtered through a 70 µM mesh strainer and the cell suspension was incubated with the CD31-conjugated Dynabeads for 1hr at 4°C. Later, CD31+ cells were separated by magnetic field using a Dynamag 2 (Thermo Fisher Scientific, Waltham, MA). Isolated cells were plated in collagen I coated 6-well plates (Corning®, Corning, NY) at a density of 3 x 10^5 cells/mL in Endothelial Cell Growth Medium 2 (PromoCell, Heidelberg, Germany) supplemented with Growth Medium 2 SupplementMix (PromoCell, Heidelberg, Germany) and Penicillin-Streptomycin-Glutamine (Thermo Fisher Scientific, Waltham, MA). The media was changed every other day and cells were passaged once reaching 70% confluence around 5 days. LECs were used at passage 2 after most beads had disassociated from the cells.

BMECs were isolated from the neonatal femurs in a sterile environment. Neonatal mice were euthanized on ice and then the hind limbs were amputated with scissors. The skin was removed

and then the femurs/tibias were sliced into smaller sections. These sections were placed in a crucible with 5mL of alpha-mem media and the marrow displaced from the femur fragments by loosely grinding the bone with a pestle. Cells displaced in the media were transferred to a 50mL conical tube fitted with a 70 μm mesh filter. The cell solution was then centrifuged, and the remaining pellet was seeded in Complete Endothelial Cell Growth Media (ScienCell, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), Endothelial Cell Growth Supplement, and 1% Penicillin/Streptomycin (ScienCell, Carlsbad, CA). Isolated ECs were plated in a 6-well plate (Thermo Fisher Scientific, Waltham, MA) in Complete Endothelial Cell Growth Media and were used at passage 1.

2.3 Subculture

All BMECs and LECs (LECs twice) were subcultured for use in experiments. Cells were lifted off of their respective plates using ACCUTASE™ (ScieCell, Carlsbad, CA), 1mL/well($1x10^6$ cells/mL). The LECs were first washed with 1mL of 1X PBS per well. Then 1mL of ACCUTASE™ at room temperature was added to each well. The cells were then placed in an incubator at 37°C and 5% CO_2 for 15-20 minutes (until detachment occurred) and then 2mL of media was added to each well to dilute the ACCUTASE™ and then transfer the cell solution into 50mL conical tubes. The conical tubes were then centrifuged at 1,200 rpm for 5 minutes. The supernatant was removed using vacuum aspiration, leaving the pellet. Then the cells were either counted or were resuspended in media and expanded from one original well to three new wells.

2.4 Proliferation Assay

Proliferation of BMECs and LECs was examined by seeding the cells in a 96-well plate at a density of $1x10^3$ cells/well. Cells were washed with 2mL/well of PBS and then 1mL of ACCUTASE™ was added to each well. The cells were incubated in ACCUTASE™ for 15 minutes at 37°C and 5% CO_2. After incubation, cells were transferred to a 50mL conical tube and centrifuged at 1200 rpm for 5 minutes. The pellet was then resuspended in 1mL of media, and a 20μL of cell solution aliquot was transferred and diluted with 20μL of Trypan Blue Solution (Sigma-Aldrich, Darmstadt, Germany). 10μL of the resulting solution was used to count cells on a hemocytometer. When the cell count was determined, cells were seeded to two 96-well plates at $5x10^3$ cells/well, and cultured

for 1 or 2 days, respectively. Cells were fixed with 5% neutral buffered formalin (NBF) at room temperature for 20 min, then stained with 0.05% crystal violet for 30 minutes. Later, cells were washed beneath tap water and left to dry at room temperature. When completely dry (several days) images were taken with EVOS® FL Cell Imaging System and cell numbers were counted using ImageJ.1.52a software. The area of the well occupied by cells was also calculated. Three representative images were captured per well.

2.5 Tube formation assay

Tube formation was assessed by Matrigel tube formation assay. Matrigel basement membrane matrix (Corning®, Corning, NY) was polymerized in 96-well plates (50µl/well) at 37°C and 5% CO_2 for 45 minutes. LECs at passage 2 and BMECs at passage 1 were plated on the polymerized basement membrane matrix at a density of 10,000 cells/well, suspended in their respective growth medium. Cells were incubated at 37°C and 5% CO_2, and images were taken after 4hr and 6hr for lung and bone marrow cells. Tube formation was quantified manually using ImageJ.1.52a software. Parameters analyzed for tube formation included the number of complete tubes, and total tube length. The number of complete tubes and the total tube length were measured manually by three independent double-blinded readers using the Simple Neurite Tracer plugin within the ImageJ.1.52b Fiji software.

2.6 Wound migration assay

BMECs and LECs were seeded in an Imagelock 96-well plate (Essen BioScience, Ann Arbor, MI) at a density of 8 x 10^4 cells/well and grown for 24 h until 100% confluence was reached. A scratch was created in the middle of each well using IncuCyte® WoundMaker (Essen BioScience, Ann Arbor, MI). Images were taken at time 0, and consecutively every 2hr, until 48hr at 10X magnification using the IncuCyte ZOOM® Live-Cell Analysis System (Essen BioScience). Images were analyzed using the IncuCyte™ Scratch Wound Cell Migration Software (Essen BioScience). The parameters for migration assessed were relative wound density (%), wound confluence (%), and wound width (µm).

2.7 Gene Expression

Table 1. RTqPCR Primers		
Gene	Orientation	Sequence (5′-3′)
CD31	Forward	ACGCTGGTGCTCTATGCAAG
	Reverse	TCAGTTGCTGCCCATTCATCA
FLK-1	Forward	AGTTGGCAACGCAGGAAAAC
	Reverse	GGGATTGACTTTGCCCCAGT
FLT-1	Forward	CCACCTCTCTATCCGCTGG
	Reverse	ACCAATGTGCTAACCGTCTTATT
ANGPT1	Forward	CACATAGGGTGCAGCAACCA
	Reverse	CGTCGTGTTCTGGAAGAATGA
ANGPT2	Forward	CCTCGACTACGACGACTCAGT
	Reverse	TCTGCACCACATTCTGTTGGA
18S	Forward	CGCCGC-TAGAGGTGAAATTC
	Reverse	CGAACCTCCGACTTTCGTTCT

Total RNA was isolated from cultured cells with TRIzol (Invitrogen Life Technologies, Carlsbad, CA). 1mL of TRIzol was used per well in a 6-well plate. A cell scraper was then used to aide in detachment and lysis of the cells. The resulting RNA was transferred to 5mL tubes and kept at -20°C until RNA isolation. Lysates were set out at room temperature to warm up. Aliquots were then centrifuged at 12,000 x g for 10 minutes, then the liquid phase was collected, and the pellets were discarded. 600µL of chloroform was added to each sample, then it was shaken briefly, and left for 2-3 minutes at room temperature. The samples were then centrifuged again at 12,000 x g for 15 minutes. The colorless upper $3/4^{th}$ of the aqueous phase was collected, then 1.5 mL of isopropyl alcohol was added to the aliquots and mixed thoroughly. After a 10-minute incubation period, samples were centrifuged at 12,000 x g for 10 minutes. The supernatant was decanted, and the pellet saved. To dissolve the RNA pellet, 3 mL of 75% ETOH was added and then the samples were vortexed. Next, they were centrifuged at 7,500 x g for 5 minutes. Supernatant was discarded, and then the pellets were transferred to a Rnase-free Eppendorf with 1mL of 75% ETOH. Samples were centrifuged for the last time at 7,500 x g for 5 minutes. The supernatant was discarded, and then the samples were allowed to dry out in the incubator before 40 µL of DEPC H_2O was used to dissolve the pellet. RNA was then stored at -80°C for later use. Before cDNA synthesis, RNA was quantified using NANODROP 2000 (Thermo Scientific, Waltham, MA USA)

The SuperScript® III First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies, Carlsbad, CA) was used to prepare cDNA from 1 µg of total RNA. An RNA/primer mix was made of 5-8µL of RNA, 1µL of Oligo(dT)$_{20,}$ 1µL of 10mM dNTP mix, and the necessary amount of DEPC-treated water to reach a total volume of 10µL. This mixture was incubated at 65°C for 5 minutes and then placed on ice. The cDNA synthesis mix comprised of 2µL of 10X RT buffer, 4µL of 25 mM $MgCL_2$, 2µL of 0.1 M DTT, 1µL of RNaseOUT™, and 1µL of SuperScript® III RT was made per sample. The 10µL of cDNA synthesis mix was added to each RNA/primer mixture and incubated for 50 minutes at 50°C. The reactions ended with a 5-minute incubation at 85°C, then the samples were placed on ice. Samples were briefly centrifuged and then 1µL of RNase H was added to each and incubated for 20 minutes at 37°C. These cDNA could then be stored at -30°C to -10°C or used.

Quantitative real-time PCR was performed using FastStart Universal SYBR Green Master (ROX) (Roche Diagnostics GmbH, Mannheim, Germany). The following primer genes were analyzed: CD31 Antigen (CD31), Vascular Endothelial Growth Factor (VEGF), Fms Related Tyrosine Kinase 1 (Flk-1), Fms Related Receptor Tyrosine Kinase 1 (Flt-1) Angiopoietin 1 (ANGPT1), and Angiopoietin 2 (ANGPT2). (Table 1). 18S rRNA primer (18S) served as the internal control. Relative gene expression was calculated using the 2-ΔΔCT method.

2.8 Femur Fracture Model

Table 2. **Surgical Groups**	
1	Young Fx + collagen sponge
2	Old Fx + collagen sponge
3	Young Fx + neonatal BEMCs/collagen
4	Old Fx + neonatal BMECs/collagen
5	Young Fx + young BMECs/collagen
6	Old Fx + young BMECs/collagen

C57BL/6 mice were used at approximately 3-4 months (young fracture group) and 26 months (old fracture group). In preparation for surgery, mice were anesthetized with isoflurane (Patterson Veterinary, Greeley, CO), and ophthalmic ointment (Major Pharmaceuticals, Indianapolis, IN) was applied to each eye. The right hindlimb was shaved and cleaned with ethanol/betadine scrubs. After a 1cm incision was made laterally over the right upper hindlimb, blunt dissection was carried

down to expose the femur, and the muscle stripped in the diaphyseal region. Next, the knee was flexed, and a 25-gauge needle was used to split the patellar tendon, the needle was then manually advanced between the femoral condyles into the femoral intramedullary canal. A sterile Dremel rotary cutting tool (DREMEL, Racine, WI) was used to remove a 2mm intercalary segment from the femoral diaphysis, and the needle was advanced through the greater trochanter. To stabilize the femur, the needle was bent onto itself and was pulled in an anterograde direction tautly against the greater trochanter. Type I collagen membranes (RCM6 Resorbable Collagen Membrane, ACE, Brockton, MA) were cut into 2cm x 1cm strips and treated with saline (control) or seeded with BMECs ($1x10^6$ cells/membrane) and were placed around the femoral diaphysis. The membrane was fixed into place with a 3-0 polyglycolic acid suture (J215H, Ethicon, Somerville, NJ). Muscle tissue and skin were then closed with 3-0 polyglycolic acid suture and standard 7mm wound clips (RF7CS, Braintree Scientific, Braintree, MA), respectively. X-ray images were used to confirm alignment of the pin/scaffold at the time of surgery and then were taken every week for 4 weeks to monitor bone healing. For 5 days after the surgery, mice were monitored daily. Old mice were kept in cages that remained on a water-heated pad during that time and were given a daily 0.5μL shot of saline. Mice were euthanized upon the final week of X-rays and the femur was harvested for uCT analysis.

2.9 Micro-computed tomography imaging

Femurs were imaged using a desktop SkyScan 1172 μCT imaging system (SkyScan, Kontich, Germany, 60 kV, 5.9 μm voxel). The callus area was scanned for analysis (60kV, 6 um resolution, 4000 pixels). The images were reconstructed using NRecon v.1.7.3. (Dyanmic Range for reconstruction was set for 0-0.1) Reconstructed images were analyzed using Bruker CT-Analyzer (v.1.15 CTAn). A Region of Interest (ROI) was determined to examine the callus area. To start the tracing process, the top and bottom of the callus region was selected. The ROI was created using 'interpolated' shape for tracing. The callus region was traced in separate cross-section images every 20 images. After tracing, the threshold range was set from 255 to 65 in 'Binary Selection Preview'. In the custom processing view, the threshold range was set as a default for all analyses. 3D analysis was run in 'batch manager'. Each bone was selected along with its corresponding ROI loaded over it. The resulting data was exported onto a text file. Reported variables include: BV, TV, BV/TV (%), Tb.Th, Tb.N, and Tb.Sp.

2.10 Tamoxifen Injections and Imaging

BMECs and LECs were isolated from Tie2CreERT+ mice around 4-6 weeks of age that had been administered 3 doses of tamoxifen via intraperitoneal injections to induce cre-recombinant Tdt expression. Tamoxifen injections were prepared at 50mg/kg in 10µL of corn seed oil. After isolation, cells were seeded on 6-well plates at $1x10^3$ concentrations and images were taken under KEYENCE BZ-X810 fluorescence Microscope (KEYENCE CORP., Itasca, IL) using Texas Red fluorescence to verify Tdt expression.

2.11 Statistical Analysis

All data are reported as mean ± standard deviation of the mean. Statistical analyses were performed using GraphPad Prism version 8.0.0 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com). Unpaired two-tailed t-tests assuming unequal variances were run to compare LEC to BMECs in tube formation path length and number, as well as gene expression for all genes. Paired two-tailed t-tests with assumed unequal variances were run to compare all three wound migration parameters. Two-Tailed Anova was run to compare LEC and BMEC timepoints and the groups proliferative capacity. Two-way anova was used to compare uCT groups for BV, TV, BV/TV, Tb.Th., Tb.N., and Tb.Sp. Significance was determined as $p < 0.05$.

CHAPTER 3. RESULTS

3.1 Tube Formation Analysis of LEC and BMECs

To determine whether LECs or BMECs show the same propensity to form vasculature, both LECs and BMECs were seeded on Matrigel matrix at $1x10^3$ concentration per well in one 96 wells plat. Cells began forming tube networks around 2 hrs and were initially imaged at 4, 6, and 8 hours, but through observation we determined that tubes peaked around 4 hours (fig 3A) and then gradually broke down from there. Two different measurements were evaluated using three blind analyzers that hand-traced the tubes on ImageJ. The number of tubes and the length of the tubes were evaluated. A two-tailed paired t-test with unequal variances was carried out, and the resulting P value was used to determine significance. A significant difference was not determined between the two cell groups for both the number of tubes (Fig 3B), and for the length of tubes (Fig 3C). The data suggests that indeed both LECs and BMECs have similar and strong vessel forming ability.

3.2 Cell Count Assays of LECs and BMECs

In this experiment cell proliferation was determined by seeding cells at $1x10^3$ per well in a 96 well plate. Three wells for day-1 and three for day-2 were seeded for each replicate on their separate plates. The cells were fixed on their respective day and stained with crystal violet (Fig. 4A). Cell proliferation was examined by comparing the counts between the two time points. This is not technically a proliferation assay, but works as an acceptable proxy to assays like bromodeoxyuridine (BrdU) that incorporate BrdU directly into replicating DNA and can be detected with anti-BrdU antibodies. [64] LECs, which we propose are an ECFC, should display a high proliferative potential. LECs indeed showed a significant increase in cell number over two days, whereas BMECs did not (Fig. 4B). BMECs did not show any significant difference between day-1 and day-2, suggesting their proliferative ability is lower, within the timeframe used.

3.3 Wound Migration

Sheet migration is a characteristic of endothelial cells and occurs in damaged tissues. [65] We plated cells at a high concentration and then made a scratch wound across the culture, allowing

cells to migrate into the wounded region over a two-day period (Fig. 5D). There were three different analytics taken into consideration: relative wound density, wound confluence, and wound width. The first is relative wound density, which compares the concentration of cells inside the wound to the concentration of cells outside the wound area. This parameter is used as a self-normalizing tool to observe changes in the cell density outside the wound created by proliferation and/or pharmacological effects. [66] LECs had a significantly higher relative wound density than BMECs (Fig. 5A). The second parameter we examined was the wound confluence, which is the cell density within the wound area. There was no significant difference in wound confluence between LEC and BMEC (Fig. 5B). The final metric tested was wound width, which is the distance measured between the wound edges as it closes. BMECs showed a significantly better migratory ability than LECs did (Fig. 5C). These data suggest that both LECs and BMECs showed an increase in migratory activity in different ways.

3.4 Angiogenic Gene Expression in LECs and BMECs

The endothelial cells were isolated using two different methods and from separate tissues. For this reason, it is interesting to see whether differences in their gene expression exist. Six different angiogenic-related genes were examined. The only significant differences observed were with Flk-1 and ANGPT2. In the case of Flk-1, BMECs had significantly higher mRNA levels (Fig. 6C). ANGPT2 showed the opposite trend with LECs having significantly higher levels of mRNA (Fig. 6F). The other four genes, CD31, VEGF, flt-1, and ANGPT1, did not show a significant difference between the two cell groups. These data suggest mostly similar gene expression profile between LECs and BMECs.

3.5 Femur Fracture Study

In this surgical model, a femur fracture was made using a Dremel surgical saw-bit and an intramedullary pin was used to stabilize the fracture. A type I collagen sponge was wrapped around the fracture site and functioned as a delivery vehicle for the three treatment groups. The three treatment groups were: a saline control, neonatal BMECs, and young BMECs. Young adult mice, 3-4 months, and old adult mice, approximately 26 months, underwent the femur fracture surgery. Only male mice were used, making the only variables age and treatment. BMECs were selected as

the cell to test in vivo. BMECs are less invasive to isolate, expand quickly, and can be used immediately upon the first passage unlike bead isolated LECs. The cells were seeded to a collagen sponge at a density of $1x10^6$ and kept in an incubator with media overnight.

After the surgeries, X-rays were taken once a week for four weeks. The images show the formation, or lack thereof, of callus formation. (Figures 8&9) Representative images were selected for each group based off observational evaluations. There were issues, specifically with the old mice, of high mortality post-surgery. A total of four old mice treated with saline died during post-operative care after the first surgery. After this, 500µL saline injections were administered every day during post-operative care (5 days) and mice were fed food that was intentionally dampened for easier consumption. After the additional precautions for aged mice, two aged mice treated with neonatal BMECs died, and one young BMEC treated aged mouse died after surgery.

After four weeks the mice were euthanized and their femurs, tibias, and contralateral limbs were harvested. µCT was performed to image the surgical femurs. None of the femurs formed bridging callus during the four-week recovery period. As a result, we could not perform a torsion testing to analyze the bone strength. We reconstructed the µCT images and analyzed the callus area using Bruker CT-analyzer.

µCT scanning provided 6 parameters: bone volume, tissue volume, mineralized callus volume, trabecular thickness, trabecular separation and trabecular number. The first three parameters give us an idea of the callus size and the amount of bone within the callus. The mineralized callus volume is the bone volume divided by the tissue volume, giving us a percentage of the callus that is mineralized. Old mice treated with neonatal BMECs displayed significantly higher bone volume than old mice treated with saline, but there was no significant difference between the other aged fracture treatment groups (Fig. 10A). Within the young mice groups there were no significant differences in bone volume (Fig. 10A). Comparing bone volume differences in age, only the difference between neonatal treated young and old mice showed significance, with younger treated mice having higher bone volume (Fig. 10A). The tissue volume of old mice treated with saline was significantly higher than both neonatal BMEC and young BMEC treated old mice (Fig. 10B). There were no significant differences in tissue volume among the young mice treatment groups.

Neonatal BMEC treated old mice were had significantly higher mineralized callus volume than young BMEC treated old mice (Fig. 10C). Although a slight increase in mineralized callus volume occurred in the neonatal and young BMEC treated young mice occurred, there was ultimately no significant difference in the young treatment groups.

Trabecular thickness of young mice treated with BMECs were significantly higher than the two other young treatment groups. (Fig. 10D) The number of Trabeculae was significantly higher in young BMEC treated old mice compared to neonatal BMEC treated old mice (Fig. 10E). The spacing between trabeculae was significantly higher in the neonatal BMEC treated groups compared to young BMEC treated groups (Fig. 10F). These data suggest that both LECs and BMECs did not enhance fracture repair in aging mice. However, regardless of either LECs or BMECs treatment, callus BV/TV, trabecular thickness and trabecular number in old mice were all significantly less than those in young mice. These data show a significant delay in fracture healing in old mice in comparison with the young mice, suggesting that we successfully establish an aging fracture healing model in mice.

CHAPTER 4. FIGURES

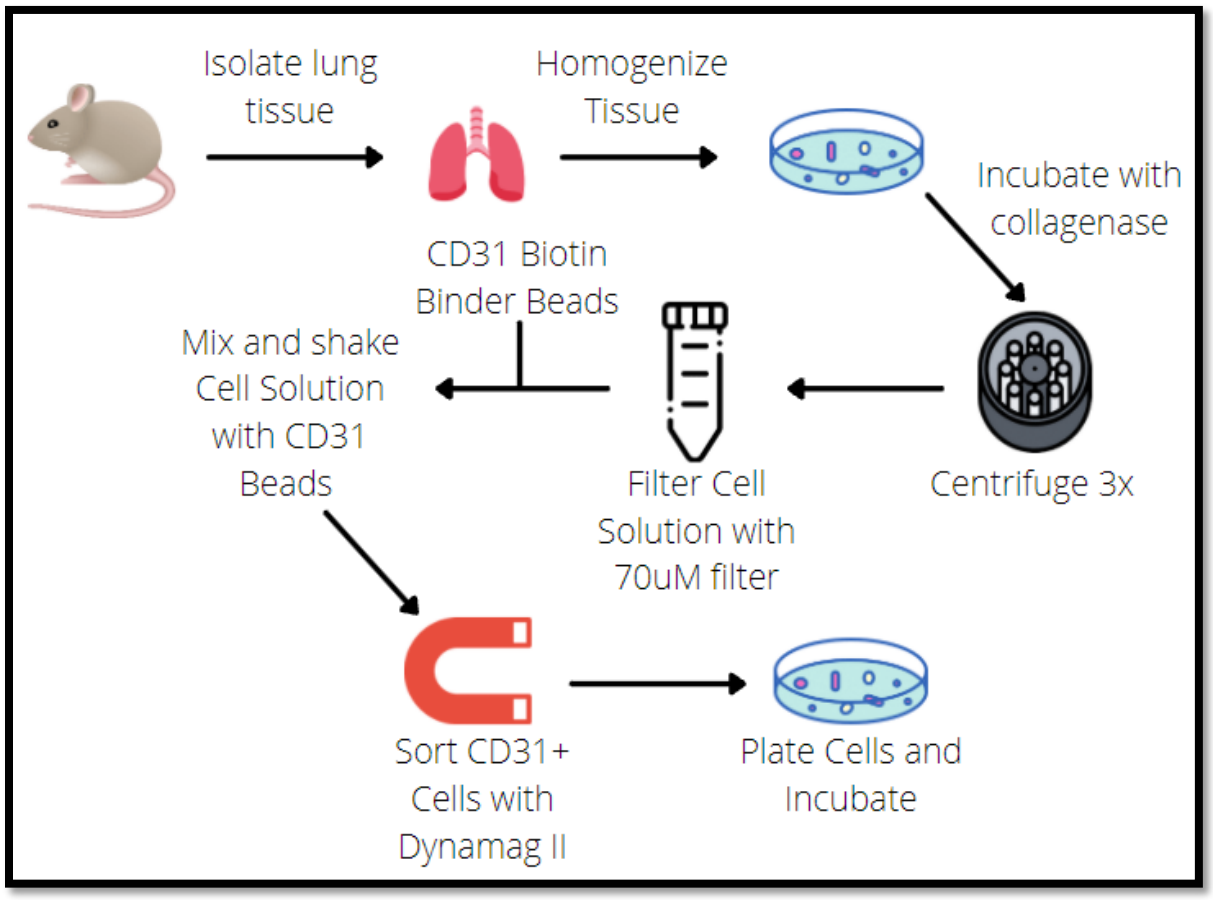


Figure 1. LEC Isolation Method

LECs were isolated from murine lung tissue. CD31 conjugated biotin binder beads were used to select LECs in a direct manner. LECs selected via CD31 biotin binder beads were separated using a Dynamag 2 and cultured on a Type I collagen-coated plate in EGM2.

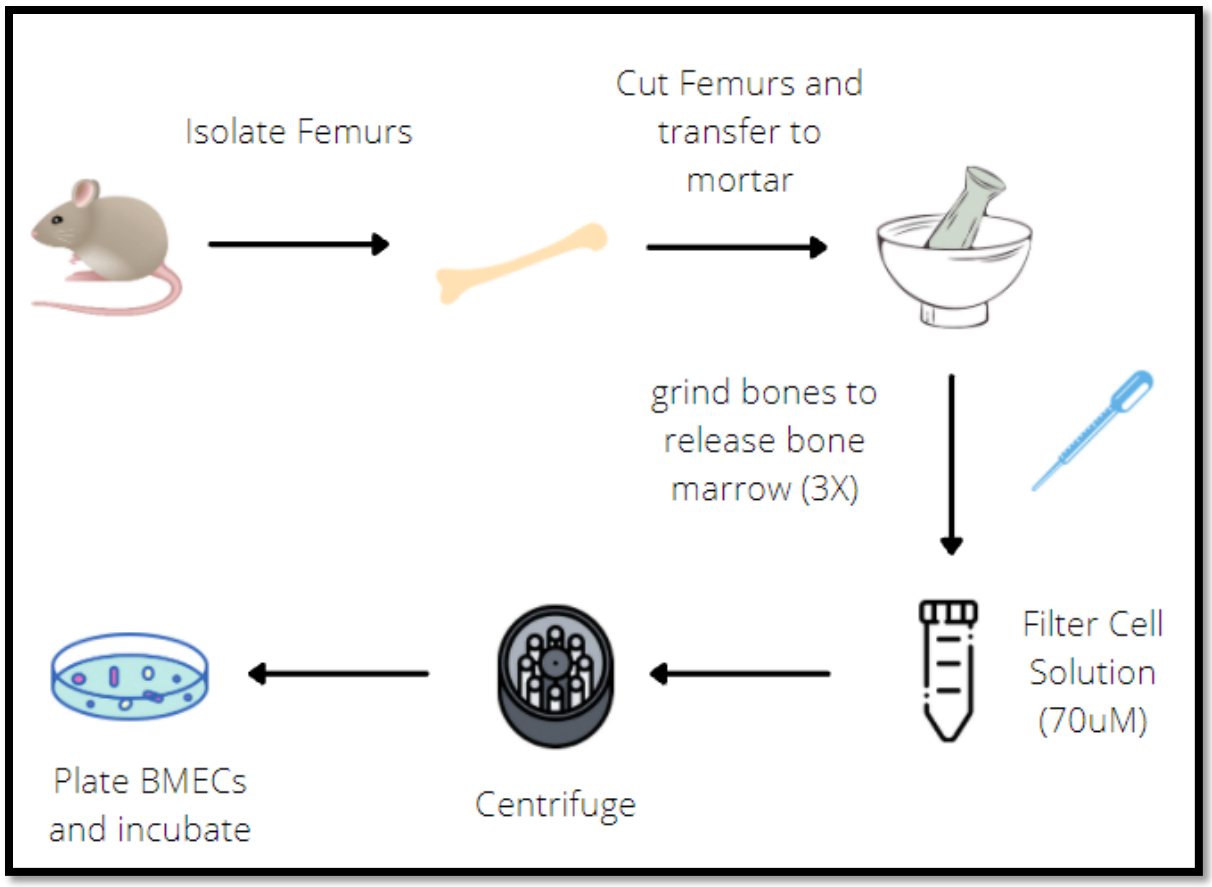
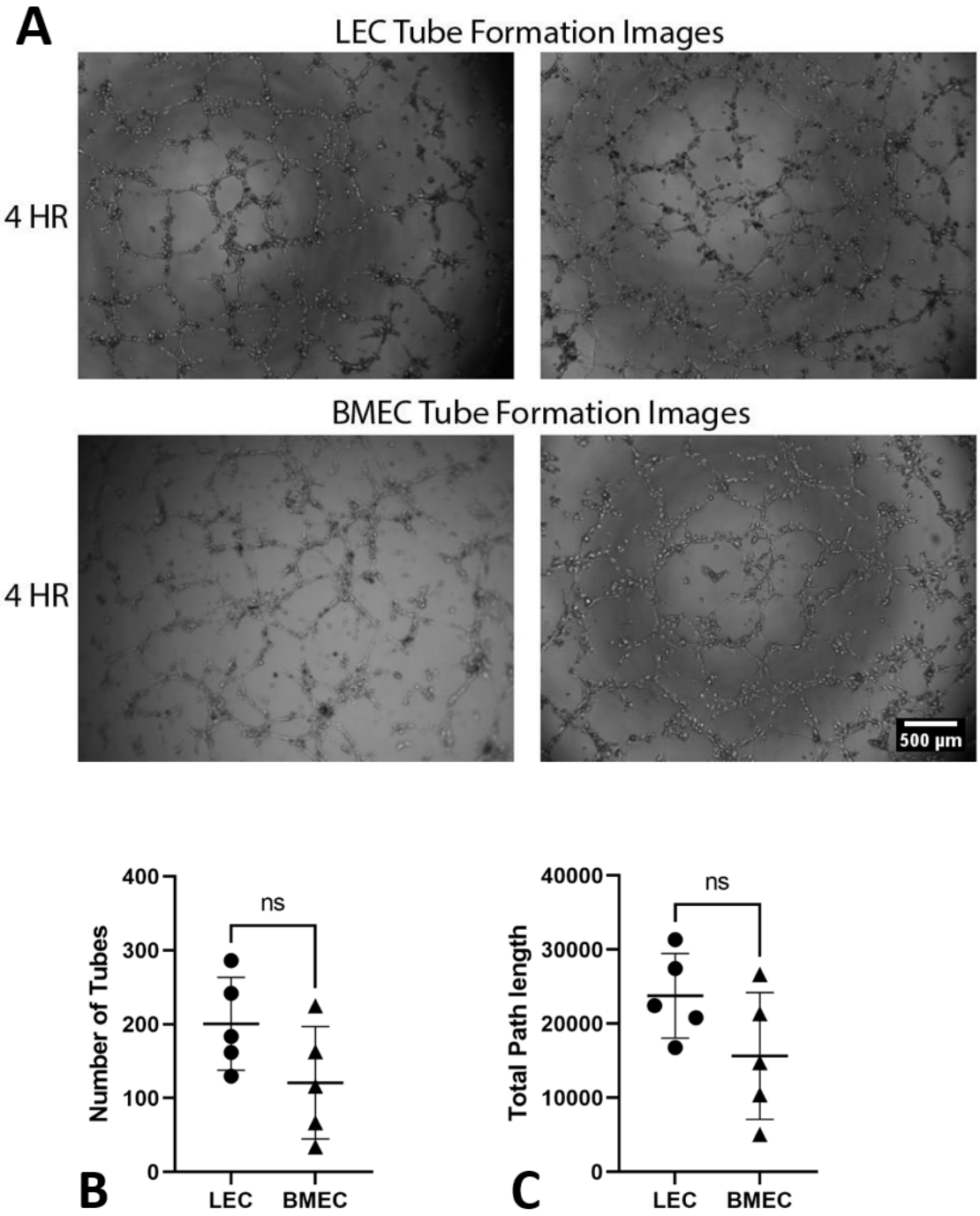


Figure 2. BMEC Isolation Method

BMECs were isolated from femurs and tibias. The bone marrow was collected by repeated pestle grinding and media washes. Between washes, media with flushed bone marrow was pipetted into a conical tube. Once all of the bone marrow had been extracted, the cells were collected via centrifugation. BMECs were plated on Type I collagen-coated wells in EGM media.

Figure 3. Tube Formation

Neonatal BMEC and LEC Tube Formation on Matrigel. LECs and BMECs were seeded on Corning Matrigel basement membrane at a $1x10^4$cell density/100uL of respective media. These images were captured at four hours of incubation. Images were analyzed using ImageJ to trace tube networks, ultimately determining the number of tubes and the lengths of individual tubes per image. (A) Two images for both LEC and BMEC tube formation assays captured at 4 hours at 4X magnification. (B) Total path numbers for each LEC and BMEC replicate. (C) Total path lengths of each LEC and BMEC replicate. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$; (n=5 replicates in triplicate/group)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LEC Tube Formation Images
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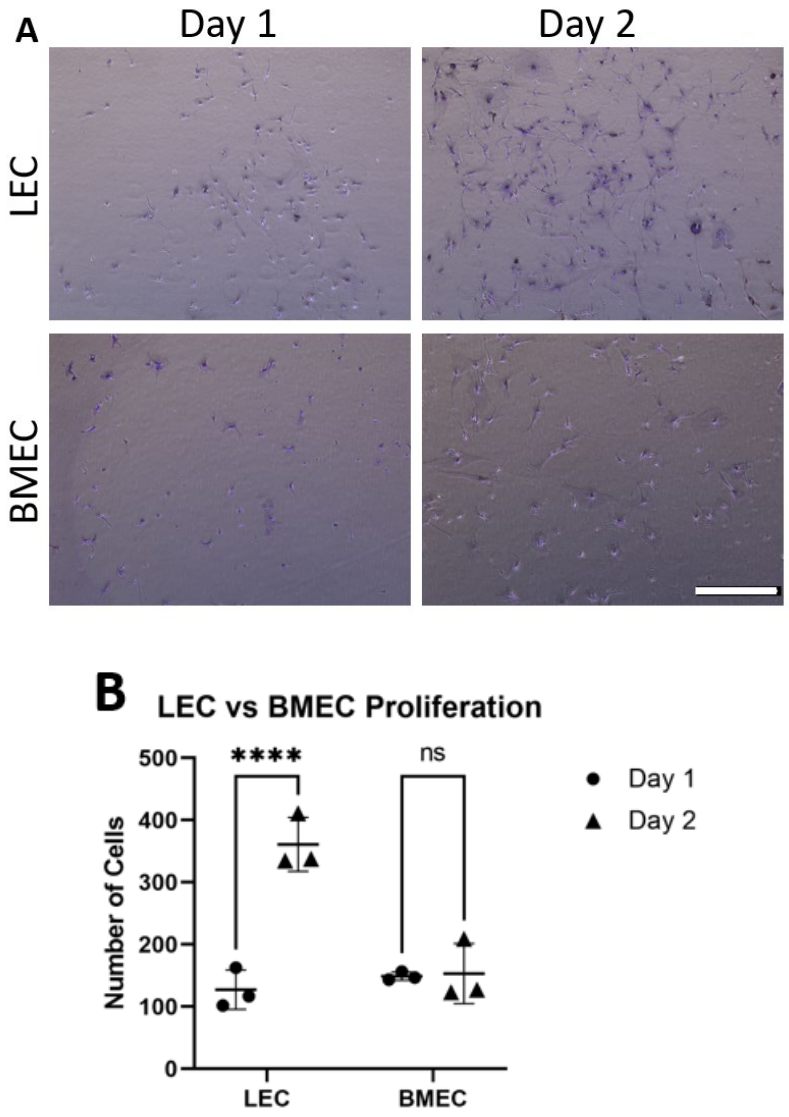

Figure 4. Proliferation Assay

Neonatal BMEC and LEC proliferation stained with crystal violet and counted using ImageJ. Cells were seeded to 96-well plates at 2,000 cells/100uL of respective media. 5% NBF was used to fix cells. These cells were later stained with 0.05% Crystal Violet and Imaged under microscope. (A) Crystal violet-stained representative images for Day-1 and -2 for both LEC and BMEC proliferation assays at 4X magnification. (B) Cell proliferation counts of Day-1 and -2 for both LEC and BMEC populations. Significance was determined by mean difference. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$; (n=5 replicates in triplicate/group); Scale bar=500µm)

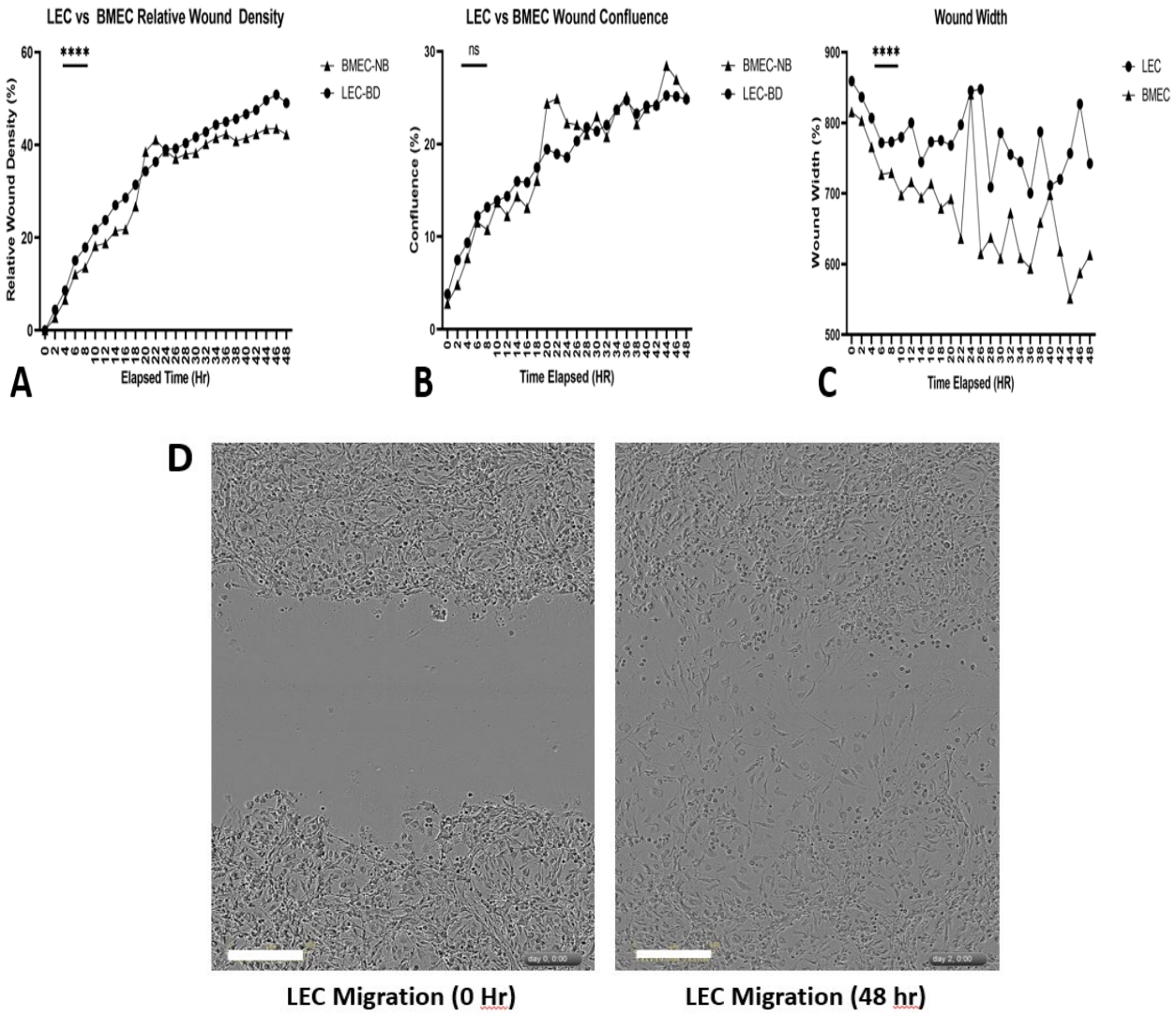

Figure 5. Wound Migration

Neonatal BMEC and LEC wound migration via scratch assay. BMECs and LECs were seeded on to 96 well plates at $1x10^5$ cell densities/100uL of EGM2 media. A scratch was made across the plate. Images were obtained every 2 hours for 48 hours. (A) Relative Wound Density, (B) Wound Confluence, (C) and Wound Width were analyzed on GraphPad using an unpaired-two-tailed t-test to determine significance. Significance was determined by mean difference. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$; (n=5; LEC/n=3; BMEC) (Scale Bar = 300µm)

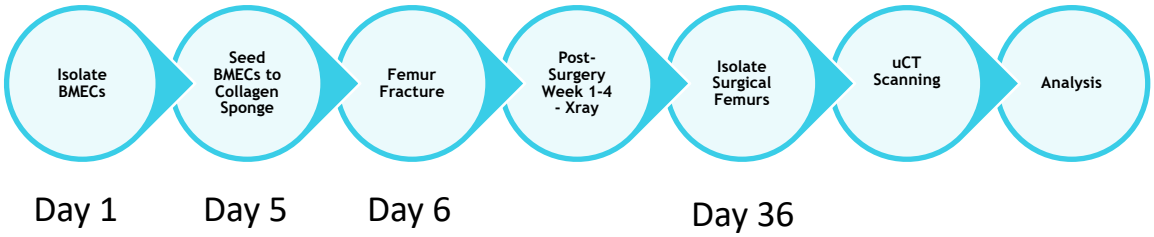

Figure 6. Fracture Study Timeline

The *in vivo* study took approximately 36 days to complete before the femurs could be scanned for uCT analysis. On day 1 neonatal and young BMECs were isolated for use in surgeries. Approximately 5 days in BMECs were seeded to type I collagen sponges. At day 6 femur fracture surgeries took place. X-rays were captured once a week for the next 4 weeks. After 36 days the femurs were harvested for µCT analysis.

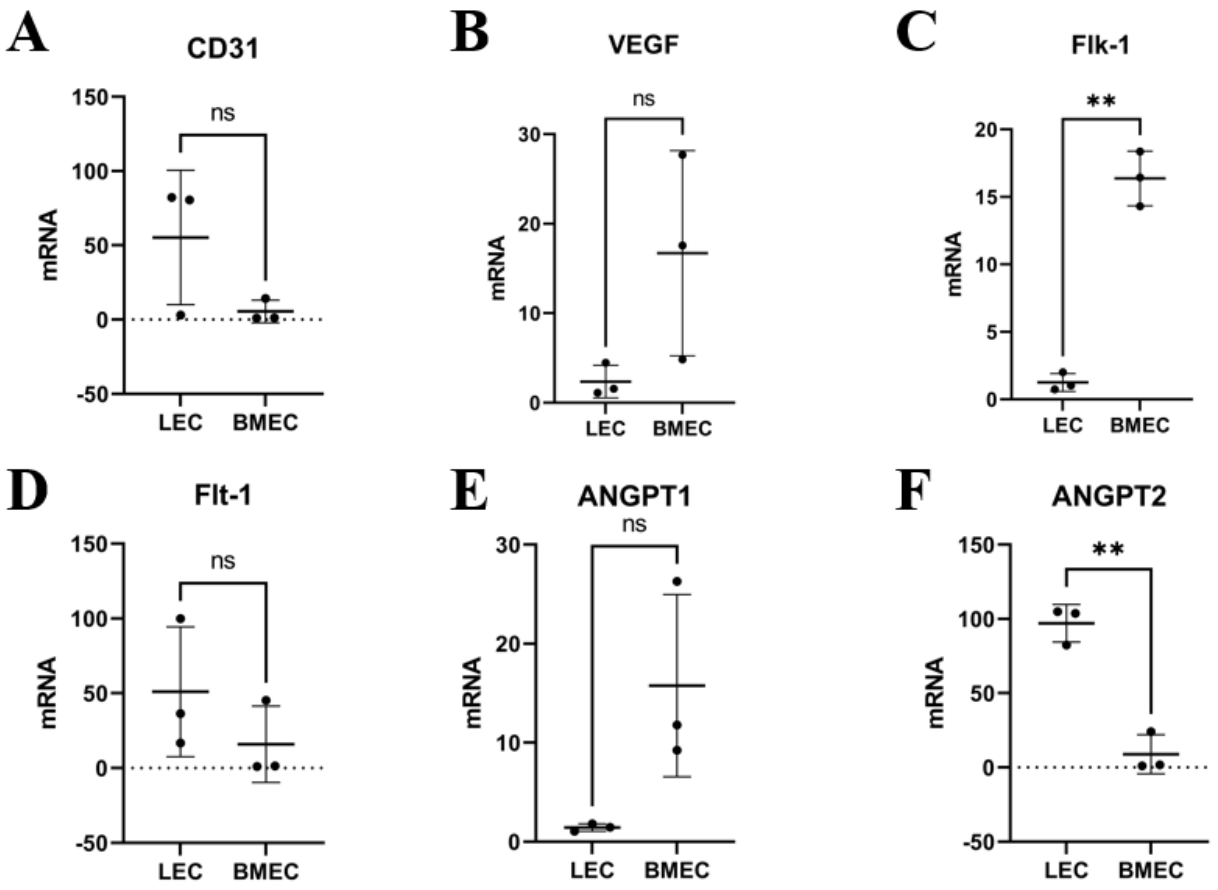

Figure 7. Relative mRNA Expression of Select Genes

Relative gene expression of (A) CD31, (B) VEGF, (C) Flk-1, (D) Flt-1, (E) ANGPT1, and (F) ANGPT2. RNA was isolated using the TRIzol method, and then cDNA was prepared using SuperScript® III First-Strand Synthesis System for RT-PCR. Finally quantitative PCR was carried out. Ct values were determined for each gene and the 2-ΔΔCT method was used to calculate relative gene expression. Significance was determined by mean difference. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$; (n=3 replicates in triplicate/group)

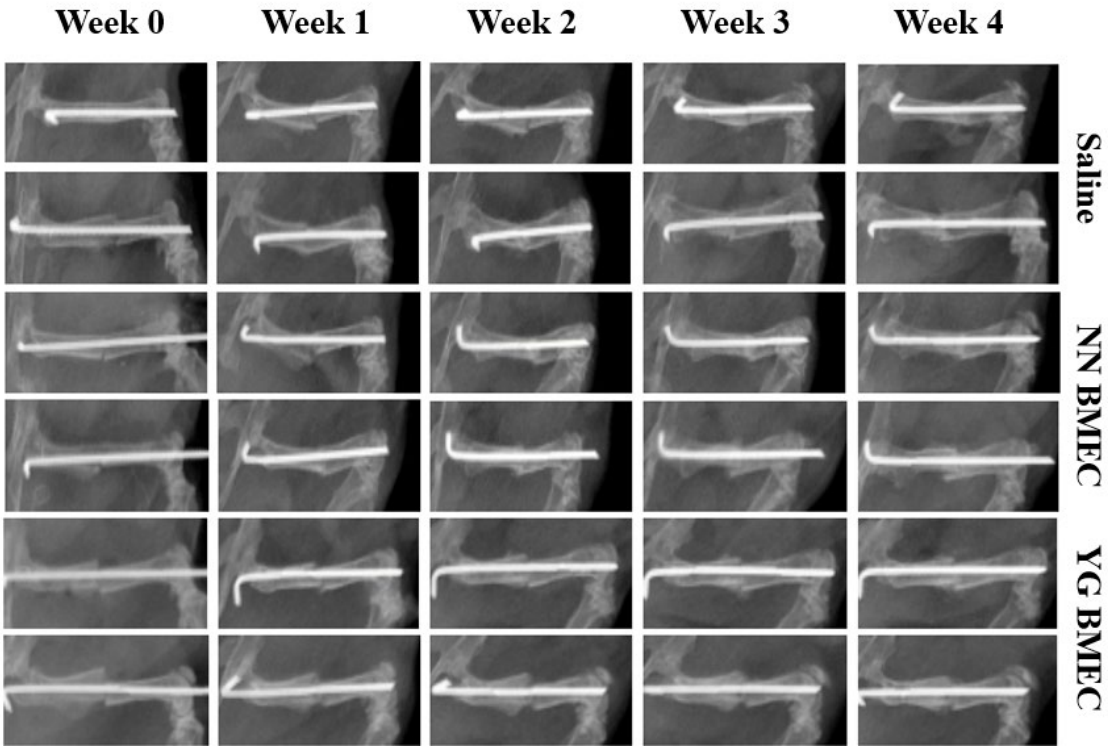

Figure 8. X-ray of Old Fracture Mice

Femur X-ray after fracture surgery in aged mice (two representative mice for each group). Femurs were X-rayed at 45kV once a week until mice were euthanized 4 weeks post-surgery. Representative X-rays for saline, NN BMEC, and YG BMEC were captured.

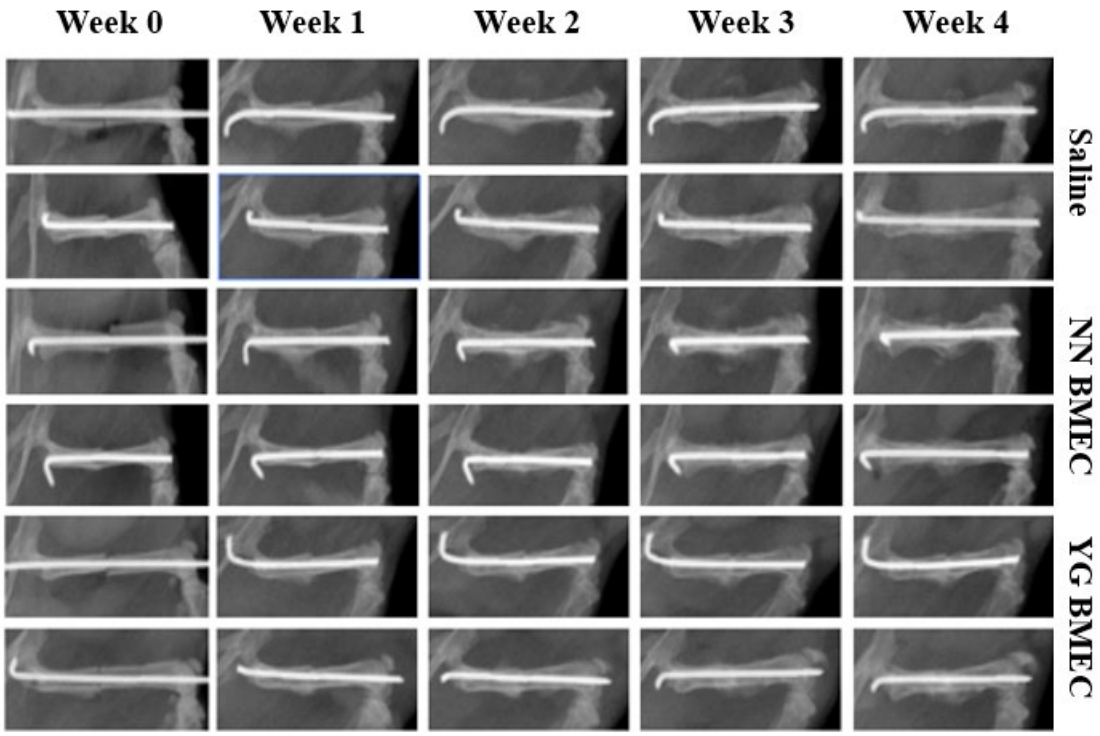

Figure 9. X-ray of Young Fracture Mice

Femur X-ray after fracture surgery in young adult mice (two representative mice for each group). Femurs were X-rayed at 45kV once a week until mice were euthanized 4 weeks post-surgery. Representative X-rays for saline, NN BMEC, and YG BMEC were captured.

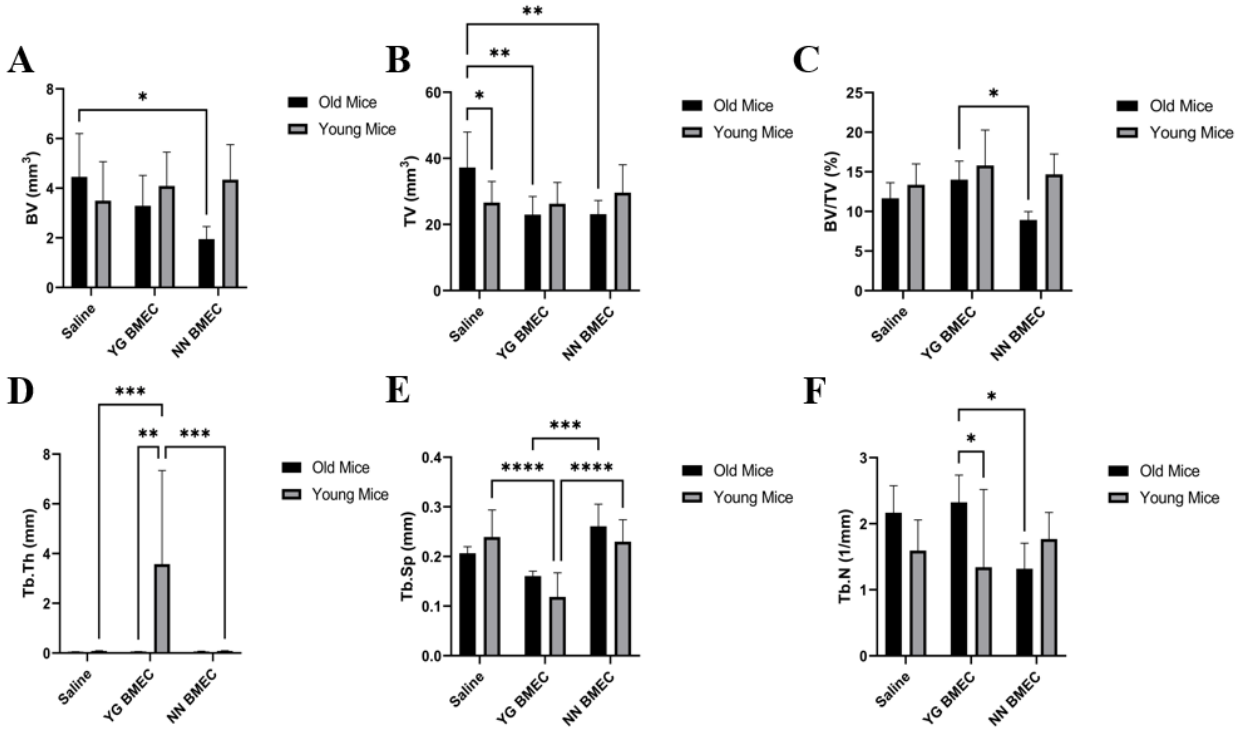

Figure 10. uCT of Surgical Femurs

Femurs were harvested four weeks after the fracture surgery. µCT imaging was performed and analyzed. The results were reconstructed and analyzed for percent callus area. (A) Bone Volume, (B) Tissue Volume, (C) Mineralized Callus Volume, (D) Trabecular Thickness, (E) Trabecular Separation, and (F) Trabecular Number were analyzed. Significance was determined by mean difference. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$; (n= 6-10)

Figure 11. 3D Fracture Models

This Figure shows the representative 3D model images of fractured femurs. One model was made per surgical group. 3D models for the three treatment groups of young mice fractures (A) and old mice fractures (B) were created.

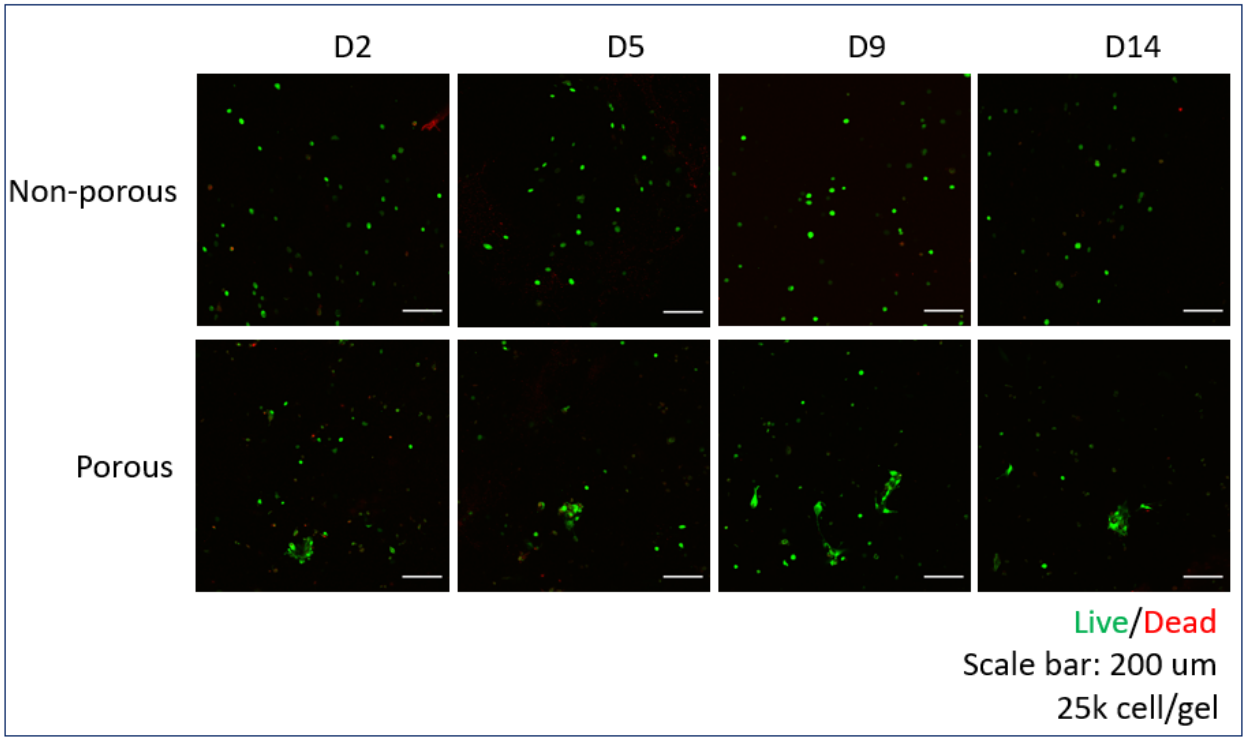

Figure 12. Hydrogel LEC Implantation

LEC cells were encapsulated in Hydrogels for 14 days. Cells in non-porous gels were compared to porous gels. Live cells are stained green and dead cells are red. These data suggest both non-porous and porous hydrogel could be a better way for delivery of endothelial cells to injury sites.

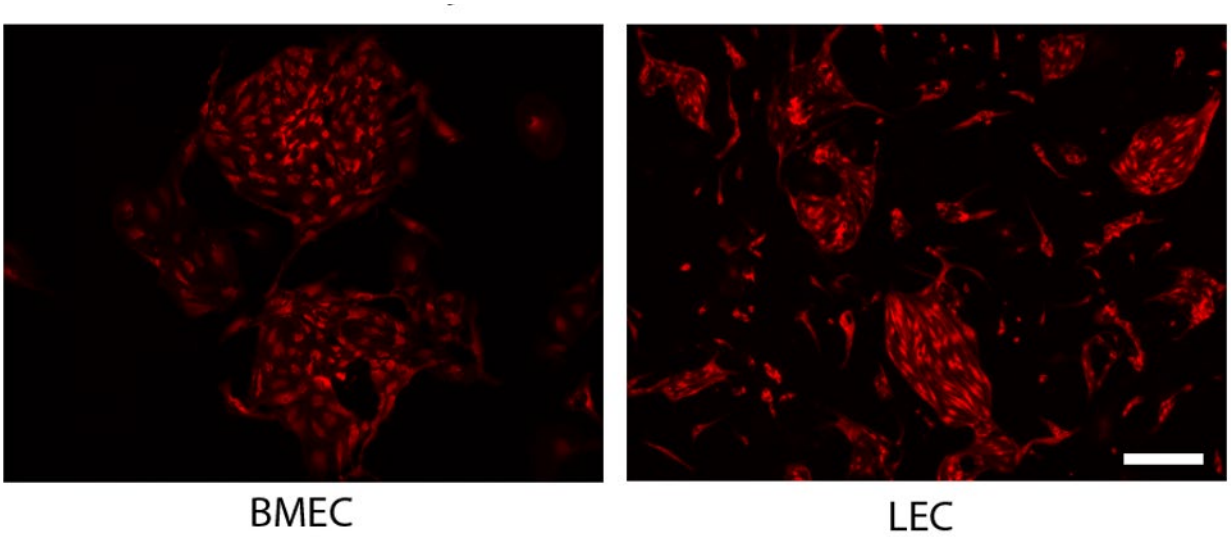

Figure 13. Tamoxifen Expression Induced via Injections

Tie2CreERT+(4-6 weeks of age) mice were given 3 injections of tamoxifen (50mg/kg) to induce in vitro expression of TdTomato. This was done in both BMEC and LEC cells, although only BMEC cells were used in surgery as a treatment. (Scale Bar = 500µm)

CHAPTER 5. DISCUSSION

Vascular invasion at the fracture site is considered an essential role in the healing process. [20, 46] EPCs have been shown to directly affect ischemic tissue repair through angiogenesis and vasculogenesis.[21, 45-47, 56] BMECs derived from femurs and tibias have been used to improve fracture healing and callus formation of critical size defects in rats. [46] Human BMECs were shown to induce vasculogenesis in myocardial infarct scars, reducing scar tissue, and keeping myocardial tissue functional, improving ventricular function in a rodent myocardial ischemic model. [22] Here we tested BMECs ability to improve bone healing in a femur fracture in mice.

Key to our study, was the use of neonatal EPC cells. Neonatal MSCs have displayed superior proliferative ability, less susceptibility to mutation, and lower immunogenicity, compared to adult derived MSCs. [59-61] Another significant advantage to neonatal EPCs, is that they are derived from non-invasively obtained tissues such as cord blood or the placenta. [60] Using progenitor cells over SCs is also an important detail. Progenitor cells are already fated to differentiate down a certain lineage, whereas SCs could differentiate away from the desired cell fate. [61] BMECs and LECs were both isolated from neonatal mice. We wanted to test whether these neonatal progenitor cells would indeed possess more potent capabilities that would lend themselves to bone regeneration. In vitro characterization was done to show the neonatal EPCs proliferative, angiogenic, and migratory ability. A genetic profile of pro-angiogenic gene expression was collected and compared as well.

This first part of the study looked at endothelial cell *in vitro* traits and the differences between BMECs and LECs isolated from neonatal tissues. Our EPC population, BMECs, were isolated by flushing bone marrow out of femurs, and then seeding those cells in an EPC specific media. The two cell populations are considered different subtypes of EPCs. BMECs are thought to be circulating EPCs. The second group of cells were LECs and were isolated using CD31 biotin magnetic beads to separate LECs into a homogenous cell culture. We performed tube formation analysis, proliferation, and wound migration experiments on the cells.

LECs belong to the ECFC population. These cells have intrinsic pro-angiogenic capabilities. They are of the endothelial lineage, and can integrate directly into vasculature, or induce de novo vasculogenesis.[21, 67, 68] In culture these cells will be highly proliferative, contain specific markers, namely CD31 in our experiment, and develop vasculature-like networks.

LECs closely resembled these phenotypes in our experiments. One difference was the appearance timeframe of LECs in culture. ECFCs typically take around two weeks to appear in culture. [54, 68] The neonatal LECs we isolated appeared a day or two after isolation. Not only did they appear more quickly, but these cells expanded rapidly, both neonatal LECs and BMECs were ready to be subcultured around 5 days post-isolation.

Focusing on LEC *in vitro* assays, these ECFCs displayed similar trends to BMECs in almost all categories. Over two days, LECs were significantly more robust proliferators than BMECs. Technically, our experiment was not a proliferation assay. The cells were seeded at low densities and then given two days to grow. Comparative counts were made of cells between day-1 and -2, and served as a reliable proxy for proliferation. Of note, LEC expansion involved the forming of colonies, that then expanded until the plate was confluent. BMECs, rather, covered the plate randomly and proliferated from there. Tube formation analysis showed that LECs were slightly higher in path number and length, but not enough to be significant. In the final trait assay, LECs ability to migrate was tested using a scratch Assay. This was important in showing that the cells could migrate into damaged tissue, as would be needed if the cells were added to a tissue scaffold. A significant difference was observed between neonatal LEC and BMEC wound widths, essentially the rate at which the wound closes. In this case, BMECs were significantly better at wound migration (Fig. 5C). Regarding varying gene expression, LECs displayed a higher expression of ANGPT2 (Fig. 7F). ANGPT2 is an important angiogenic factor that regulates vascular stability. [69] Endogenous ANGPT2 will act as an antagonist of ANGPT1 in ECs. [63] The tyrosine kinase receptor Tie2 is ANGPT2s major receptor. [69, 70] Higher expression of ANGPT2 may suggest the LECs are important in vascular remodeling and are more sensitive to environmental stressors.

BMECs share endothelial-like characteristics in culture, such as CD31, though literature suggests that these cells act more in a paracrine role than by directly incorporating into vasculature. [54] These cells are important for consideration because of their vascular reparative ability, but also because they can be harvested easily, and yield many cells for expansion *in vitro*. Additionally, these cells also show capable proliferative and tube forming ability in culture. In the case of migratory ability, BMECs showed significantly better wound migration ability (Fig. 5C), lending further support to BMEC use in bone regeneration. LECs required an extra passage to decrease the residual magnetic beads in culture, whereas BMECs can be used immediately upon the first

passage. BMECs had significantly higher Flk-1 expression than LECs (Fig. 7C). Flk-1, also known as VEGF receptor 2 (VEGFR2), is a tyrosine kinase receptor that binds VEGFs. [71] Flk-1 activation influences angiogenesis and vasculogenesis through endothelial cell proliferation, migration, promoted survival, and differentiation. [71] Flk-1 has a lower affinity for binding VEGF-A, the major pro-angiogenic VEGF, but much higher signaling activity than VEGF receptor 1 (VEGFR1) once bound. [71, 72] Despite having significantly higher Flk-1, there was no significant difference in Flt-1 between the cell types. LECs are still reactive to VEGF signaling, however, BMECs higher expression of Flk-1 suggests they more actively influence blood vessel development.

LECs are isolated specifically with CD31, a requisite ECFC marker, making their population homogenous. The BMECs are comprised of a heterogeneous population that may have stalk and tip cells, ECs, parenchymal cells, and EC-like cells. [73] These differences may influence the cells response in the assays performed. Pericytes for example, have been shown to act as vasculature progenitor cells, promote new vessel formation, and recruit other pro-angiogenic cells in models of myocardial ischemia. [61] Another interesting consideration is that a heterogeneous population of cells would retain more of their natural traits, being in a close proxy environment for their niche in the body. Historically, most EPCs have been isolated from bone marrow and blood. More recently, vasculature and tissue resident EPCs are coming into the picture. Better understanding the stem cell nice for EPCs and MSCs within vasculature and tissue could be very important to providing information on ischemic tissue repair and also a way to collect the most potent EPCs for use in tissue regeneration.

In the second part of the study, we tested BMECs potential to improve bone repair within an induced femur fracture. The nominal hypothesis, do EPCs improve bone healing, was unsubstantiated by the data. This, however, seems to be more an issue of experiment design, and not simply a failure of the theory. The older C57BL/6 that were given EPCs did not show an increase in bone formation compared to their saline counterparts, in fact, they displayed less bone formation. Old mice treated with the saline control had significantly higher tissue volumes than old mice treated with neonatal and young BMECs (Fig. 10B). Regarding bone volume, old mice treated with saline had significantly higher bone volumes than neonatal BMECs (Fig. 10A). The mineralized callus volume of old mice treated with young BMECs was significantly higher than neonatal BMECs, but not the saline-treated old mice (Fig. 10C). Young mice fared slightly better

but did not show any significant improvements in bone, tissue, and mineralized callus volume compared to the saline-treated young mice (Fig. 10C). Altogether there was no significant improvement observed from the use of BMECs. Since the bones did not achieve union in the four-week healing period, other factors may have influenced the healing process.

This data suggests that age did indeed play a role in the body's ability to react. BMECs act upon the bone healing process in a paracrine method. The cells can act directly upon the vasculature by differentiating and incorporating into the tissue to grow the vasculature but appear to operate more in a paracrine role. In the paracrine model, they release GFs, such as VEGF, that signal the residential endothelium to form new vessels. In our experiment, it is possible that the BMECs were indeed successful at engrafting to the injured region, but the residential endothelial cells were unable to properly respond to the paracrine signaling. This may be even more prevalent in the aged mice, where the cells would have senescence derived inefficiencies. Mutations build up steadily in adult SCs over time, which could potentially lead to phenotypic changes in adult SCs that disrupt regenerative abilities. [74] Young mice treated with neonatal BMECs showed significant increases in bone volume and mineralized callus volume when compared to old mice treated with neonatal BMECs (Figure 10).

Our characterization tests did show EPCs propensity to form vasculature-like structures, to proliferate in vitro, and to migrate efficiently. Along with that, cells can be harvested quickly, and the isolation methods yields strong populations of cells that expand expediently. These factors suggest that EPCs remain a strong choice for tissue regeneration. Clearly changes to the surgical model need to be made. A new scaffold needs to be selected. We have already looked at a possible transition to a patterned hydrogel scaffold. These gels are porous, allowing for vasculature to grow through them and for cells to migrate through them. Importantly, they should breakdown quickly and allow the tissue to heal properly without impeding the healing process.

In addition to using a type I collagen sponge to seed cells to for implantation, BMECs should be directly transferred to a scaffold. We have already investigated the possible use of patterned hydrogels as a potential scaffold for bone healing. These scaffolds would satisfy the main requisites for bone regeneration. Patterned hydrogels are popular scaffolds for potential use in BTE. They are composed of highly hydrated polymeric networks, making them well equipped for tissue engineering and drug delivery. [37] We collaborated with Dr. Chien-Chi Lin in the BME department, where they have developed their own patterned hydrogel scaffold. Neonatal LECs

were able to successfully adhere and survive within a patterned hydrogel in culture (Figure 13). They were seeded in both porous and non-porous gels, and a significant increase in survival was observed in the porous gels (Figure 12). Cells were incubated up to 14 days, showing that the LECs may be cytocompatible with these gels as well.

Another improvement would be to track BMECs in vivo by inducing cre recombination with tamoxifen injections. We injected Tie-2CreER;Td-tomato mice around 4-6 weeks of age with 50mg/kg of tamoxifen. BMECs and LECs were isolated from these mice and plated for imaging. Red fluorescence was activated via tamoxifen induction of the cre recombinase system (Figure 13). This tool can be used for in vivo stem cell tracking to check for EPC engraftment at the fracture site.

In conclusion, LECs and BMECs used in vitro showed a strong propensity for tube formation, proliferation, and migration, all needed for proper vessel development to affect tissue regeneration. We have developed a delayed fracture healing model in aged mice. While we are looking into the possibility of using a hydrogel for the delivery of the ECs to the fracture site, we can use this fracture model to test other potential drugs or stem cells for enhancement of bone healing.

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