

**ENHANCEMENT OF ANKLE FUSION THROUGH FK506
INDUCED OSTEOGENESIS**

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by

Nicholas Huffman

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ENHANCEMENT OF ANKLE FUSION THROUGH FK506 INDUCED OSTEOGENESIS

Approved by:

Dr. Jay Patel, Advisor
School of Biomedical Engineering
*Georgia Institute of Technology &
Emory University*

Dr. Johnna Temenoff
School of Biomedical Engineering
Georgia Institute of Technology

Dr. Jason Bariteau
Emory School of Medicine
Emory University

Dr. Edward Botchwey
School of Biomedical Engineering
Georgia Institute of Technology

Date Approved: [December 08, 2023]

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LIST OF SYMBOLS AND ABBREVIATIONS

BMP	Bone Morphogenic Protein
PDGF-bb	Platelet Derived Growth Factor (BB isoform)
BMDC	Bone Marrow Derived Cells
ALP	Alkaline Phosphatase Assay
BSA	Bovine Serum Albumen
μ CT	Micro-computed Tomography
MDC	Marrow-derived cells
FBS	Fetal Bovine Serum
OA	Osteoarthritis
BV	Bone Volume
TMD	Tissue Mineral Density
hMSC	Human Mesenchymal Stem Cells
FDA	Food and Drug Administration
TV	Tissue Volume

SUMMARY

Ankle Arthrodesis is a common surgical procedure that typically involves the fusion of the tibia and talus of the patient. During surgery, the surgeon uses screws and plates to compress the bones together and cease plantar and dorsiflexion motion [1]. However, one of the main complications with the surgery is the non-union of bones. This can be due to loosening of the screws or failure to grow new bone in the joint space. Our team hypothesized that introducing an additional orthobiologic into the system would assist in bone formation and reducing non-union rates. In this study, we evaluated the effectiveness of osteogenic drugs to improve bone fusion within ankle arthrodesis. One such molecule we evaluated is FK506 (Tacrolimus), an FDA approved drug for treating organ transplant rejection. We implemented a cell culture model to test out the osteogenic potential of FK506. Bovine Marrow Derived Cells (MDCs) were cultured for 1-2 weeks and evaluated with Alizarin Red S Staining, Results were also tested with hMSCs. ALP Activity, and Gene Expression. We found that FK506 significantly affects Alizarin Red S staining within our MDCs. Additionally, we identified that rhPDGF-bb could be a potential adjuvant to FK506 treatment. Though future work will be needed to confirm the effects of rhPDGF-bb within an in vivo model. It was also noticed that there was significant variation associated with the MDC results between donors. We will look to answer those questions with flow cytometry in future experiments. Following those results, we tested our model within a rabbit ankle model to evaluate effectiveness.

CHAPTER 1. INTRODUCTION

Osteoarthritis is a condition that affects around 3.4% of the general population [2]. The condition can range in severity and can develop at a young age following injury to the surrounding cartilage. OA is caused by the breakdown of cartilage to expose the underlying bone, this results in inflammation and swelling of the area [3]. Surgical treatment usually falls within three different categories: arthroscopy, arthrodesis, and arthroplasty [4]. Arthroscopy is where the surgeon creates an incision into the joint and removes loose cartilage and shaves bone spurs in the area. The goal is to reduce friction in the joint space that can make OA worse and delay physical progression of the disease [5]. Arthrodesis is the fusion of the joint and is typically done with screws and plates to hold together articulating bones. Arthroplasty involves the complete replacement of the joint with a prosthetic. Arthroscopy is typically a preventative treatment to temporarily relieve pain, while arthrodesis and joint arthroplasty are more permanent options [6]. However, joint arthrodesis is associated with a high non-union rate [7]. Thus, new approaches are needed to improve the healing rate of this procedure.

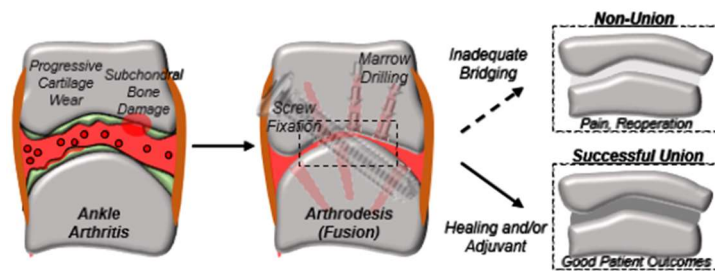


Figure 1. Overview of Ankle Arthrodesis treatment. Arthroscopy of the joint space followed by screw fixation and marrow drilling can lead to functional clinical outcomes.

1.1 Background

1.1.1 Ankle Fusion

One of the most common procedures for treating end-stage osteoarthritis of the foot and ankle is arthrodesis. Arthrodesis is also a cost-effective solution for patients, typically costing \$20,000 less than total ankle arthroplasty [8]. The surgery involves an initial arthroscopy of the joint space to remove cartilage and bone spurs followed by screw and/or plate fixation depending on how osteoporotic the bone is [9]. The main joint involved within the fusion is called the tibiotalar joint. This is where the tibia connects with the talus to connect the leg and foot [10]. The tibiotalar joint is responsible for plantarflexion/dorsiflexion and inversion/eversion of the ankle [11]. So, immobilization of this joint is crucial to the success of the surgery. Ankle arthrodesis can be done through the open and arthroscopic method. Open arthrodesis allows for easy application of plates and correction of malalignment. Consequently, open arthrodesis is a more invasive approach and is associated with a higher incidence rate of surgical complications [12]. The arthroscopic approach is becoming more and more popular in the US as it is less invasive and can be used for immunocompromised patients [1]. However, there is no clear decision regarding the effectiveness of using one approach over the other as both procedures lead to similar fusion rates.

1.1.1.1 Problems in the Field

One of the main problems with ankle arthrodesis is the non-union rate of surgery. This can be as high as 12 % and can result in additional surgeries or rehabilitation if the

fusion is incomplete [13]. Additional surgeries can be costly and cause unnecessary pain for the patients.

Non-union within arthrodesis defined as the inability for bone to heal and mend within the joint space [13]. This is typically confirmed through radiograph and can help identify other issues within the fusion. There are numerous reasons why non-union occurs following arthrodesis: infection, poor patient blood flow, biomechanical instability, and general patient biology [14]. Infection is a common topic within the medical device field as this can cause aseptic loosening of the medical device. Biofilms of bacteria can form on screws or plates and this can cause issues for the patient [15]. Poor blood flow of the general area can inhibit migration of immune cells to the area and other osteoblasts/bone forming cells to help healing the arthrodesis site. This can also result in osteonecrosis if oxygen is not being delivered to the injury site [16]. Since the procedure is typically done on end-stage osteoarthritis patients, it is common for the patient to have poor healing compared to a healthy patient. Smoking, local infection, and limited vascularity are some of the main risk factors for decreased fusion [13, 14]. In addition to this, physical therapy is an essential factor in eventual union healing. Incorporation of weight-bearing protocols into patient recovery plans is imperative. It has been shown in clinic that loading the injury site leads to better healing outcomes compared to non-loading [17]. This parallels bioreactor design, where mimicking the mechanical environment of the cell can lead to enhanced differentiation compared to general cell culture methods [18]. Matziolis et al. has shown that BMSC cells within a

cyclic compression load have shown enhanced osteogenesis compared to traditional culture methods [19].

Ankle arthrodesis surgery is typically followed by a lengthy rehabilitation time in order for the joint to fully heal [20]. Patients will wear a non-weightbearing cast to limit post-operative pain and radiographs will be taken periodically to confirm the fusion. Recovery times can last up to 1 year and is accompanied by significant changes in gait biomechanics [21]. This can affect day to day activities until the patient learns how to walk with the fusion. Apart from walking, this can also interfere with other aspects of daily life. It has been shown that patients with ankle fusion surgery exhibit decreased brake reaction timing when driving [22]. This is one of the many considerations that must be taken into account before surgery.

1.1.1.2 Primary Need

Around 25,000 ankle fusions are performed each year in the US [23]. With an average cost of around \$7900 per surgery, this a roughly \$200 million USD industry [8]. However, rising costs due to non-union revision surgery and other quality of life adjustments can deter patients from choosing this surgical option. Thus, changes are needed to improve the surgery to assist patient satisfaction and surgical effectiveness. In order to do this, we must refine the fixation and introduce new methods.

1.1.1.3 *Current Methods for Fixation*

1.1.1.4 Mechanical Fixation

In order for an arthrodesis surgery to be successful, the joint needs to be completely immobilized while the ankle is allowed to heal. This can be done through both external and internal methods of fixation.

1.1.1.4.1 External Fixation

The use of external fixators is a more complicated approach that is reserved for patients with extremely poor bone quality, and large bone defects. There are multiple types of fixators: Hoffman external fixator, Calandruccio clamp, thin wire ring fixators, and hybrid fixators [24]. One of the challenges with these types of fixators is the risk of infection. Since the pins are anchored to the bone and connected to an outside metal frame, the articulation point where the rod meets the skin is extremely prone to infection. The pin site is an active wound site that cannot heal until the fixator is removed. The external fixator also requires additional upkeep and support since it takes up space around the limb. This can affect patient compliance when choosing between internal and external fixation.

Though this seems like a major complication, it has been shown that external fixators induce less disruption to the periosteum and blood supply to the bone than internal methods [25]. Additionally, if the patient case is so severe where internal fixation cannot be utilized, external fixation can provide similar fusion rates.

1.1.1.4.2 Internal Fixation

Internal fixation is considered the gold standard to mechanical fixation. This is usually achieved by a combination of screws, plates, and intramedullary nails. Screws are the most common tool used for internal fixation because of the ease of use and cost associated with the procedure [1]. If the patient has a large area of viable bone, then screws are the primary option.

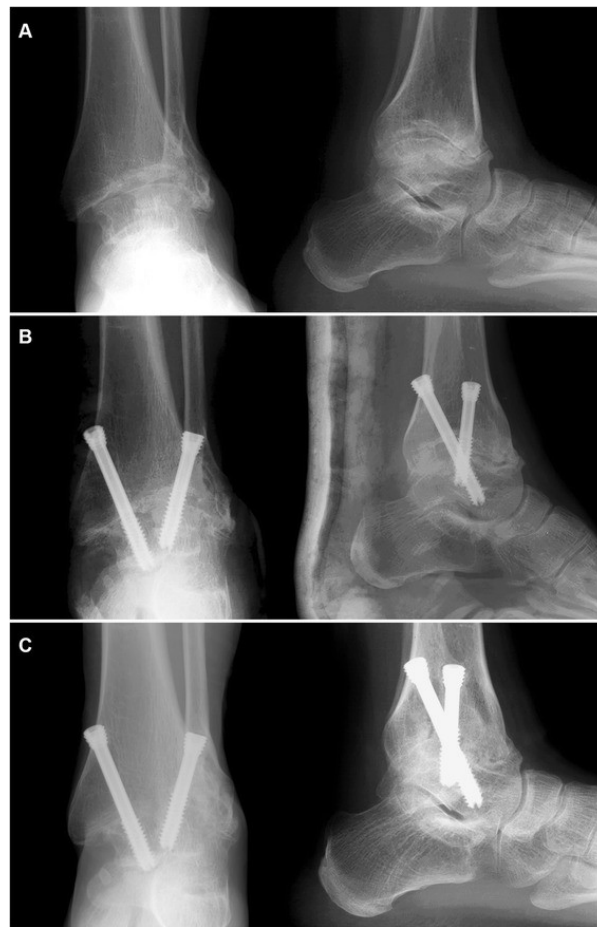


Figure 2. Visualization of Arthroscopic Ankle Fusion. Crosses transfiguration of cannulated screws. Adapted with permission [26] via Creative Commons Attributions License 4.0 International License. Copyright © 2016, Journal of Orthopaedic Surgery and Research

However, if the patient is osteoporotic or there is not a lot of healthy bone available the surgeon will use plates. Plates can come in either conventional or locking styles, which depend on the location and the overall bone health. Plates have been shown to increase union rates, but they also have a higher rate of infection due to the surgical area required to place the plate [1]. Plates have more surface area than screws, which can lead to potential biofilm formation. An advantage of using plates is the constant compression provided compared to screws. Locking plates distribute compressive forces equally to remove stress concentration areas.

1.1.1.5 Biological Fixation

In addition to mechanical fixation, surgeons are incorporating biologics into their approaches to aid fusion [27]. Biologics can come in many forms such as growth factors, scaffolds, and cells. It is thought that adding biologic cues in addition to mechanical stimulation can aid fusion rates.

There are two types of biologics currently approved by the FDA for use in fusion sites: osteoconductive scaffolds and growth factors. Scaffolds such as autografts, demineralized bone matrix, hydrogels, and other mineralized pastes can assist with cell integration. They allow for cell adhesion and can provide mechanical support. Growth factors can stimulate osteogenesis and chemotaxis of stem cells to the fusion site. By allowing the migration and differentiation of stem cells into osteoblasts, bone formation can be increased and improve non-union rates. Another important aspect of biologics is angiogenesis within the fusion site. Formation of blood vessels is imperative to healing

outcomes and is important in the first weeks of osteogenesis [28]. Blood vessels allow for cells to migrate into the fusion site and provide oxygen and nutrients to the growing tissue. This is especially important within an older patient population that may not have good vasculature.

1.1.1.5.1.1 Autologous Bone Grafts

Autologous bone graft (ABG) must be osteoconductive and osteoinductive to allow for bone healing. ABG's can be either cancellous bone, cortical bone, or a combination. The main objective of incorporating an ABG is to provide mechanical stability along with patient osteogenic and angiogenic cells for bone healing. Providing a structure for cells to fill in and create vasculature can assist and guide the healing process for a better healing outcome. ABG's also contain the patient's own cells and growth factors for inducing healthy bone growth. In addition to this, the ABG tissue cannot be rejected by the patient compared to an allograft.

The donor site location is of extreme importance when it comes to harvesting a graft. This can depend on patient age and other underlying conditions. For ankle fusion grafts, one of the most popular graft spots is the calcaneus and the tibia [29]. This is mainly due to the location and availability of the bone during the procedure. Though the two locations are less ideal than the iliac crest, they can still provide an osteogenic scaffold for the patient. One of the main complications associated with the ABG is donor site morbidity. The harvest site can become infected and cause pain for the patients at the harvest site. Using bone grafts in older patients with delayed healing must be done

cautiously since the quality of the bone may be poorer and the healing time is increased. Thus, age can be a large risk factor for the graft procedure.

1.1.1.5.1.2 Synthetic Bone Grafts

Another strategy that a surgeon can use is to implement synthetic bone grafts within the grafting procedure. These bone grafts are designed to be osteoconductive to allow for bone to grow onto the scaffold [29]. Some common examples of synthetic grafts are Calcium Sulfate (CS), Tricalcium Phosphate (TCP), and Demineralized Bone Matrix (DBM). CS is available in both putty and powder form and is rapidly resorbed within the body within six weeks [30]. CS demineralizes surrounding bone which can release BMP's into the local environment to stimulate bone formation. Although CS is one of the most common bone grafts, the rapid resorption time diminishes its use as a potential void filling scaffold.

TCP is a ceramic biomaterial that has similar mechanical strength to cancellous bone. For treatment methods that require more structural support, TCP is a great candidate. It also has a high degree of porosity to match natural cancellous bone within the body. This is optimal for filling bone defects as it allows for cells to infiltrate and populate the matrix [30]. While TCP has not been studied extensively within ankle fusion, the material is used commonly within fracture and long bone repair.

DBM is manufactured from discarded bone by demineralizing and decellularizing it to create a final organic 'putty' to insert into the surgical space. The isolation process of removing the cells and mineral leaves osteo-inductive factors such as BMPs and other

proteins to stimulate growth [31]. Since it is patient derived, there is variation in terms of protein content per DBM batch. There are even questions of whether the protein leftover is viable since common sterilization techniques (gamma radiation, gas plasma, etc.) can potentially denature the proteins [30]. Nonetheless, DBM is an attractive candidate for bone remodeling compared to traditional synthetic grafts due to the incorporation of growth factors.

1.1.1.5.2 Orthobiologics

If the surgeon determines that taking a bone graft is not viable, they can use alternatives such as osteogenic biologics. Biologics are very easy to implement within the surgical procedure and can help mimic the osteogenic environment. These biologics typically represent growth factors that can aid in creating endogenous bone in the local environment.

1.1.1.5.2.1 RhPDGF-BB

RhPDGF-BB is a 24.kDa protein that is approved by the FDA under the name Regranex © to treat diabetic ulcers in the foot and leg. PDGF-bb is one of five different isomers, but it can uniquely bind to every membrane-bound PDGF receptor. Thus making PDGF-bb an attractive candidate for research on top of its mitogenic and chemotactic properties [32]. It is administered as a topical gel to deliver the growth factor locally. rhPDGF-BB stimulates angiogenesis and initiates capillary formation within the wound site. While rhPDGF-bb does not have a direct effect on osteogenesis like the BMP family, it is thought that introducing a pro-angiogenic agent to allow for chemotaxis and migration of

MSC cells to the injury site can improve overall healing [33]. This provided the foundation for the incorporation of rhPDGF-bb into treatment options.

Limited vascularity is not the only issue to osteogenesis within the joint space. Other risk factors such as diabetes, tobacco use, and anti-arthritic medicine use can delay or offset the natural healing cascade. Another consideration that surgeons must take into account is the patient's age. It has been shown that younger patients (age 18-59) who have undergone an ankle arthrodesis achieve significantly higher fusion than older patients (age 60+) [34]. Higher age in patients is accompanied by lower bone mass density, decreased vascularity, and osteoarthritis. Thus, rhPDGF-bb seems like the ideal candidate to overcome these obstacles.

DiGiovanni et al performed a randomized control trial where patients selected for ankle fusion were given either a rhPDGF-bb/Beta-TCP composite or autograft prior to arthrodesis to evaluate fusion. They found that the rhPDGF-bb/Beta-TCP resulted in comparable fusion rates and fewer side effects than the autograft [33]. This result is seen as ideal for older patients as autograft treatment is accompanied by adverse side effects. This work led to the FDA approval of AUGMENT (Wright Medical) as the first class III clinical treatment for ankle and hindfoot arthrodesis. Zhang et al has also shown through lentivirus induction of PDGF-bb, that there was significantly increased alizarin staining and ALP activity compared to control [32].

1.1.1.5.2.2 Bone Morphogenic Proteins

Bone morphogenic proteins (BMP's) are some of the most widely studied molecules in orthopedic research. There are over 20 types of BMP's within the body, however only BMP-2 and BMP-7 are on the market [29]. Because of the natural bone-stimulating properties of this growth factor, multiple treatments have been developed with its use. One of the main issues surrounding BMP pharmaceutical treatment is the high dosage required to initiate clinical bone formation. Preclinical studies showed that higher concentrations of BMP-2 are needed in more developed species [35]. The presence of noggin, a BMP inhibitor, requires more BMP to be delivered to overcome the inhibition. The combination of these two observations results in milligram level delivery dosages required to achieve clinical bone formation [36]. Combining BMP-2 with controlled release strategies such as 3D layered scaffolds is a promising future directive for BMP-2 treatment.

Medtronic has developed INFUSE ©, an FDA approved autograft replacement for open tibial fractures. INFUSE consists of an absorbable collagen sponge that is soaked with rhBMP-2. Because of INFUSE's success within bone growth, it has been approved for off-label use within interbody spinal fusion procedures [37]. However, this off-label use has resulted in serious complications regarding reports of cervical spine swelling [38]. This called for the FDA to issue a black box warning in 2008 for the product. INFUSE can also result in ectopic bone growth and heterotrophic ossification within the spinal fusion site. In an area like the vertebral column, any ectopic bone formation and swelling can result in serious complications. Outside pressure on the spinal column can result in

damaged nerves and lesions of the area. Nervous tissue regeneration is extremely slow, so any injury can have large setbacks. This makes the ankle joint such a unique case for this treatment. Due to the anatomy of the joint compared to the spine, ectopic bone formation is not as severe of a side effect.

Hoang Nam Dang, et al organized a pilot study in 2020 investigating the effect of a BMP-2/HA scaffold within an ankle fusion in vivo model. They used a rabbit model and injected solutions of rhBMP-2/HA (0.04 mg/kg) into the ankle joint prior to screw fixation. This treatment was compared to autologous bone (harvested from the distal femur). The study found that the rhBMP-2/HA group showed a statistically higher union percentage compared to the autologous bone and control [39]. Histological analysis revealed that there was better osteointegration in the rhBMP-2/HA group as well. A PubMed search for ‘Ankle Arthrodesis + Rabbit + Arthrodesis’ revealed 2 studies performed. This shows the novelty of the study design and gives us an opportunity to advance the knowledge of ankle fusion within a rabbit model.

BMPs continue to be used within clinical treatments, however there is considerable doubt regarding the safety and effectiveness of BMP treatment. Thus, research into alternative factors that can exhibit the same level of osteogenesis is of great interest to the field.

1.1.2 Gaps and Potential Treatments

1.1.2.1 FK506

FK506 (Tacrolimus) is a hydrophobic antibiotic isolated from *Streptomyces tsukubaensis*. FK506 is an immunosuppressive drug that inhibits calcineurin activity and T cell proliferation [40]. The primary mode of activation is by suppressing T cell signaling of NFAT, which impairs IL-2 production. IL-2 is critical to the differentiation and maturation of T cells [41]. FK506 is part of the FK binding protein (FKBP) family and binds to FKBP12. FKBP12 serves as an intracellular BMP inhibitor and binds to prevent 'leaky' signaling of BMP during low binding concentrations [42]. This secondary signal works in conjunction with Noggin to block both extracellular and intracellular BMP signaling. The FKBP12 binding complex inhibits BMP type 1 receptor phosphorylation, the addition of FK506 prevents the binding complex from forming [41]. This can allow for spontaneous ossification, which sparked the push for FK506 as a potential osteogenic therapeutic target. However, ectopic bone formation is not listed as a side effect for systemic delivery of Tacrolimus [mayo clinic]. This sparks some possible explanations: FK506 needs a BMP stimulus to create bone growth, systemic administration does not deliver the right concentration to induce bone growth, or noggin inhibition must supersede FKBP12 inhibition.

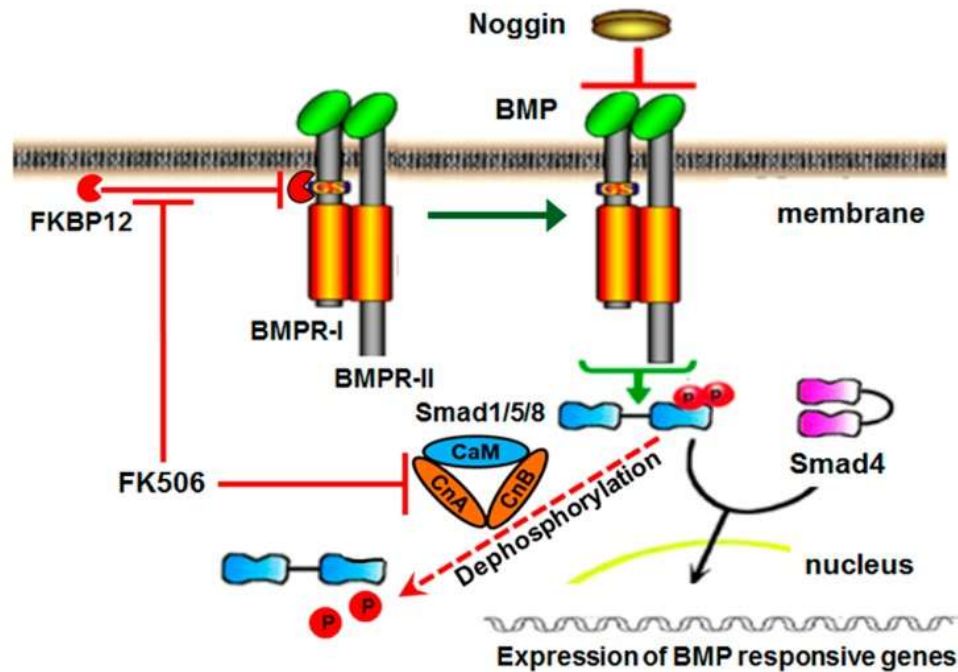


Figure 3. Bone Morphogenic Protein signaling pathway and inhibition by Noggin. Adapted with permission [42] via Creative Commons Attributions License 4.0 International License. Copyright © 2019, International Journal of Molecular Sciences

Previous research has demonstrated that FK506 can act as a stand-alone molecule capable of initiating osteogenesis and bone formation [42]. Sangadala et al subcutaneously implanted an FK506-loaded collagen sponge and found that FK506 exhibits similar bone formation compared to BMP-2. This work suggests that FK506 is a safer alternative to BMP-2 treatment for ectopic bone formation. FK506's effect on fracture healing was investigated by Voggenreiter et al using a rat tibia model. FK506 was delivered systemically at 1mg/kg for 14 and 28 days respectively [43]. They found that FK506 did not have any effect on fracture healing through radiograph analysis. Kaihara et al studied the temporal effects of FK506 in an intramuscular rat model. They

injected FK506 (1mg/kg/d) following lyophilized collagen disc implantation with BMP-2. Samples were sacrificed on 7, 14, and 21 days. It was found that the tissue calcium content was increased compared to the control in days 7 and 14 but decreased in day 21. This suggests that long-term FK506 administration can negatively affect bone formation [44]. FK506 also appears to have different effects when delivered systemically versus local delivery. More research is needed into localized FK506 delivery for osteogenesis research.

1.2 Specific Aims

The objective of this study is to evaluate and explore alternative osteogenic molecules to BMP-2 and their effect within a representative ankle fusion environment.

Our hypothesis is that the addition of FK506 to an ankle fusion environment will increase osteogenesis markers compared to the control within MDC and hMSC cell populations. Previous work done by Sangadala et al has shown this effect within C2C12 cells. This work will serve to expand the role of FK506 and investigate the effect on a clinically representative cell population.

To test this hypothesis, I developed both an in vitro 2D model and an in vitro 3D model.

The aims are as follows:

1.2.1 Specific Aim #1

Investigate FK506-induced osteogenesis in marrow-derived cells and human mesenchymal stem cells.

1.2.2 Specific Aim #2

Evaluate FK506-induced osteogenesis within a 3D culture system.

Overall, the aims above help to diversify applications within FK506 bone healing and to give a new direction for research into this molecule. Using the marrow derived cells within the in vitro culture gives insight into a heterogenous cell population and initiates the question about personalized medicine. Future directions will include investigating the effect of FK506 within an in vivo rabbit model. Additionally, we would like to investigate different cell populations of patients with high risk factors versus low risk factors.

CHAPTER 2. MATERIALS AND METHODS

2.1 Cell Culture

2.1.1 *Bone Marrow Derived Cells*

Marrow-derived cells (MDCs) were isolated from juvenile bovine femoral condyles. To do so, cubes of bone marrow were rinsed vigorously in heparin media (0.2% w/v), and the resulting solution was plated on a 48cmx48cm culture dish to obtain adherent mononuclear cells. MDCs were incubated for one day and then the cell media was changed. These cells were expanded in basal media (DMEM supplemented with 1% PSF/10% FBS). The basal media was changed every two days and cells were monitored to reach confluence. Once cells had reached confluence, the plate was washed with sterile 10mL 1xPBS and incubated for 8 min with 0.05% Trypsin/EDTA solution (Thermo-Fisher) at 37 degrees Celsius. The trypsin solution was then neutralized with 17mL basal media and the cell concentration was counted using a hemocytometer.

2.1.2 *Human Mesenchymal Stem Cells*

One million Cryopreserved Human Mesenchymal Stem Cells (hMSCs) isolated from human bone marrow were purchased from RoosterBio. Cells were verified to be tri-lineage and surface marker expression analysis verified the cell type. Upon receiving the cells, the cells were split into two 48cm x 48cm culture dishes to obtain a confluent monolayer. Cells were expanded in alpha-MEM media supplemented with 10ng/mL FGF-2. Media was changed every two days. Cultures were expanded into passage 3 or 4

until around 20 million cells could be harvested. The cells were harvested using a 0.05% Trypsin/EDTA solution at 37 degrees Celsius for 8 min. Cells were counted and centrifuged at 300g for 5 min to obtain a cell pellet. The remaining cell solution was then placed into cryovials seeded at 1 million cells per 1mL vial within a freezing solution (90% FBS, 10% DMSO). The cryovials were then stored at -20 degrees Celsius for 1 hour followed by 24 hours at -80 degrees Celsius to then move into the liquid nitrogen tank.

2.1.3 Fibrin Gel Formation

We decided to use a 96-well plate as our mould for our fibrin gels. The wells were treated with 100uL of sterile Pluronic F-68 (Thermo-Fisher) for 30 min prior to making the gels. During this time, cells were harvested using trypsin and counted using a hemacytometer. The cells were then re-suspended in 1xPBS and centrifuged at 300g for 5 min to obtain a cell pellet. The aspirate was discarded, and the pellet was concentrated down to get 5 million cells per mL of gel solution. A 100uL gel was prepared with: 10uL Thrombin (100IU/mL stock), 10uL CaCl₂ (200mM stock), 30uL cells/1XPBS. The gel solution was prepared in a sterile 1.5mL tube and then mixed gently. 50uL of the gel solution was added to each well and then 50uL of Fibrinogen (60ug/mL) was added to the well to form the gel. This solution was mixed gently to ensure no bubble formation. The gels were then incubated at 37 degrees Celsius for 45 min to allow for gel formation. After 45 min, the gels were removed from the well plate and placed into untreated 24-well plates for 3D culture. Aprotinin (1:1000 IU) was added to each well to prevent gel degradation during 3D cultures. The final fibrinogen concentration within the gel is 30mg/mL.

2.2 Biochemical Assays

2.2.1 *Alizarin Red*

Cells were plated within 24 well plates at the following concentrations: 10,000 cells/well for MDCs and 20,000 cells/well for hMSCs. The cells were allowed to culture overnight in basal media (DMEM, 10% FBS, 1% PSF). Following 1 day of culture, the cell media was replaced with 1mL of treatment media. Treatment media is denoted here: Basal Media (DMEM, 10% FBS), OM (DMEM, 10% FBS, 10mM B-glycerophosphate, 0.1uM dexamethasone, 50ug/mL ascorbic acid), OM Supplement (1:4 ratio of Basal Media, MesenCult Osteogenic Differentiation 5x supplement). Cell media was replaced every two days with new treatment media. The cultures were taken out to either 7 or 14 days to evaluate the stain. Once the timepoint was reached, the cells were washed with 500uL of 1xPBS and fixed in 10% formalin for 30 min at 4 degrees Celsius. Following the formalin fix, the wells were washed 3x with DI water to remove formalin. After the 3rd rinse, 0.5mL of Alizarin Red S (VWR, 470300-106) was added to the wells slowly in order to not disrupt the monolayer. The well plate was then incubated for 20 min at room temperature to ensure staining of the monolayer. The dye was then aspirated and washed 3x with DI water to remove non-specific staining. After, 0.5mL of DI water was added to the wells to keep the cells from drying out, samples were imaged using EPSON photo scanner to visualize the plate.

Quantification of the alizarin intensity was done through cetylpyridinium chloride elution. A 100uM solution of cetylpyridinium chloride (VWR, USA) was made in DI

water and 0.5mL was pipetted into each well. The alizarin solution was allowed to solubilize for 1 hour at room temperature to form a purple solution within the well. The well plate was then analysed at 570nm to quantify the staining using a Microtek Plate Reader.

2.2.2 Alkaline Phosphatase Assay

MDCs or hMSCs were plated at 100,000 cells/well in 6-well plate and 20,000 cells/well in 24-well plate. The cells were cultured overnight in basal media (DMEM, 10% FBS). After overnight culture, the treatment medium was added and allowed to culture until the timepoint. Once the timepoint had been reached, the cells were washed with 1xPBS and lysed with 0.5mL 0.2% Triton X-100 solution in DI water. Each well was pipetted up and down to ensure equal mixing and lysing of the cells. The plate was then incubated for 20 min at room temperature. Following the 20 min incubation, the cell lysate was collected into 2.0mL microcentrifuge tubes and vortexed. A 96-well plate was obtained and 50uL of each sample was transferred into the plate. From the ALP Assay Kit (Sigma-Aldrich), the working reagent was prepared and 150uL was added to each well to make 200uL total volume. The solution was gently mixed and then covered with aluminium foil. The plate was immediately read at 405nm at 0 min and then at 15 min to obtain the difference values.

Protein count was evaluated using the Bio-Rad microtiter plate protocol (Bio-Rad, USA). A new 96-well plate was obtained and 10uL of each sample was pipetted into the well along with standard curve protein samples (BSA). Next, 200uL of the dye reagent was

added to the well plate and allowed to incubate for 5 min at room temperature. The absorbance was then read at 600nm through the plate reader. Standard curve and protein count values were determined through the interpolated values. ALP activity was normalized to the protein amount (nmoles of p-nitrophenol per ug protein).

2.2.3 Live/Dead Assay

Live/Dead analysis was performed on both MDCs and hMSCs to determine effects of the pharmacological treatments. Cells were seeded at 50,000 cells per well (25% density) in a 24 well plate. The cells were then incubated overnight in basal media to allow adherence to the plate. The media in the well was aspirated and the treatment media was added to the wells for 48 hours. After 48 hours of treatment the Live/Dead viability assay kit (Millipore Sigma, USA) was used to evaluate cytotoxicity. Cell monolayers were imaged using the ECHO Revolve microscope to visualize live cells (Calcium AM) and dead cells (Propidium Iodide). Treatment concentrations were analysed and compared to the controls.

2.3 Cryosection Staining

After a 2-week culture within the 3D Fibrin gels, the gels were washed with 1xPBS and then fixed for 3 hours within a 10% formalin solution. The gels were then placed within a plastic cryosection mould and embedded in OCT freezing medium (VWR, USA). The gels were frozen overnight and then cryosectioned into 20uM sections. The sections were collected on glass microscope slides for visualization and staining.

2.3.1 *Collagen-1 Staining*

For staining, the OCT was rinsed off using 1xPBS, soaking for 5 min before aspirating off. A proteinase K solution (1:10000 dilution) was added for 5 min to digest the sections. The samples were then blocked for 30min with a 1% BSA solution (Millipore Sigma). The samples were then stained with the primary antibody (Collagen-1 monoclonal antibody anti-mouse, 1:200 dilution) for 60 min at room temperature. The samples were then rinsed three times in 1xPBS and stained with the secondary antibody (488 goat anti-mouse IgG, 1:200 dilution) for 60 min at room temperature. The samples were washed three times with 1xPBS. One drop of ProLong Gold with DAPI (ThermoFisher, USA) was added on each sample and a glass coverslip was placed overtop. The samples were visualized using the ECHO Revolve microscope (ECHO Laboratories, USA) using the FITC setting. Images were overlaid with the DAPI filter to determine cell patterns.

2.3.2 *Alizarin Red S Staining*

The OCT was rinsed off using DI water, soaking for 5 min before aspirating off. A wax ring was circled around the sample using Liquid Blocker (Electron Microscopy Sciences, USA). Alizarin Red S solution (VWR, USA) was added to each ring and allowed to incubate for 5 min. Following the incubation time, the sample was washed 3 times with DI water. The samples were dehydrated with 95% Ethanol twice and then with 100% Ethanol twice. All ethanol was aspirated off from the sample and Xylene was added to the sample to clear the tissue. The sample was incubated for 5 min at room temperature

before removing the xylene. Samples were then mounted with Permount mounting medium (Electron Microscopy Sciences, USA) and covered with a glass coverslip. Samples were imaged using the brightfield setting on the ECHO Revolve Microscope (ECHO Laboratories, USA).

2.4 Nuclear Localization

In order to investigate the nuclear localization of osteogenic transcription factors, we decided to use a chamber slide model. Cells were seeded at 1000 cells/well on an 8-well glass chamber slide (Nunc Lab-Tek, USA). Cells were fed with basal media and allowed to adhere overnight before adding the treatment media. After three days of treatment, the cells were washed with 1xPBS and fixed in 10% formalin for 10 min at 4 degrees Celsius. The formalin was removed, and the chamber slide was washed three times with 1xPBS. The cells were permeabilized with 0.5% Triton X-100 for 5 min and then blocked in 1% BSA for 1 hour. The slide was then stained for the primary antibody (RUNX2, D1L7F, Rabbit mAb #12556, 1:200 dilution) for 1 hour at room temperature. The cells were then washed three times with 1xPBS and stained for the secondary antibody (Phalloidin 647 with goat anti-rabbit IgG 488). The sample was incubated at 1 hour room temperature before washing again three times with 1xPBS. A final stain with DAPI (1:1000 dilution) was added to the chamber slide for 10 min at room temperature and washed three times with 1xPBS. One drop of ProLong Gold Antifade (Thermo-Fisher, USA) was added on each sample and a glass coverslip was placed overtop. Quantification of the nuclear intensity and cytoplasm intensity was done using ImageJ software.

2.5 Gene Expression

2.5.1 RT-qPCR

Real-time qPCR was done to determine potential temporal effects of the osteogenic treatments. Collection varied based on either a monolayer or 3D gel extraction:

Monolayer: 1mL of TRIzol reagent (Invitrogen, USA) was added each well of the 24 well plate. The sample was pipetted up and down in order to mix the sample and incubated for 5 min at room temperature.

Gel: Gels were transferred to a 2mL microcentrifuge tube and 1mL of TRIzol reagent (Invitrogen, USA) was added to each of the tubes. The gels were then homogenized using a tissue homogenizer (VWR, USA). The solution was homogenized until there were not any visible chunks within the TRIzol solution. The sample was then incubated for 5 min at room temperature.

Monolayer or gel samples were transferred to a 2mL microcentrifuge tube and 0.2mL of chloroform was added to the tube. The samples were incubated for 2-3 min and then centrifuged for 15 min at 12,000g x 4 degrees Celsius. Roughly 600uL of the clear layer was transferred to a new microcentrifuge tube without touching the organic layer. 600uL of 70% Ethanol was then added to the clear layer and vortexed to mix the sample. The sample was then transferred to a spin cartridge (Invitrogen, USA) and RNA was isolated according to the manufacturer's protocol. 250ng of RNA was obtained per sample for the cDNA synthesis. PowerUp SYBR Green Master Mix (Thermo Fisher, USA) and qscript

cDNA was used for the PCR analysis. All primers were designed by Integrated DNA Technologies for both bovine and human samples.

Table 1: Gene Primer List for rt-qPCR Expression

Gene Name	Forward	Reverse
B-RUNX2	CGGAGTGGAAGAGGCAAGAG	GGATGAGGAATGCGCCCTAA
B-IBSP (Bone Sialoprotein)	GCATGGCTACTTTTATCCTGC	CTTCATTCCCTCCATTGTTCC
B-BGLAP (Osteocalcin)	GACCACATCGGCTTCCAG	GGAGTTTATTCTAGAGCAGCGG
B-Osterix/Sp7	CAACCTGCTGGAGATCTGAG	AGGGAAAGATGGATAGAGTTGC
B-Osteopontin/OPN/SPP1	GTGATTTGCTTCTGCCTCTTG	AAAGTCTGCTTCTGAGATGGG
B-COL1A1	AAAAGAGGCACGCTGGTAC	GTGGTAGGTGATGTTCTGGG
B-ALPP/ALPI	CAAACCGAAACACAAGCACTC	GCATTGTTCTGTTGAGTTCG
B-ACTB β -actin	GGCATTACGAAACTACCTTC	TGATCTCTTCTGCATCCTGTC
H-ACTB	ACCTTCTACAATGAGCTGCG	CCTGGATAGCAACGTACATGG
H-IBSP	ATTTCCAGTTCAGGGCAGTAG	GTGTGGTATTCTCAGCCTCAG
H-Runx2	TTCACCTTGACCATAACCGTC	GGCGGTCAGAGAACAACCTAG
H-OPN	CAGTGATTTGCTTTTGCCTCC	ATTCTGCTTCTGAGATGGGTC
H-OCN	CAGCGAGGTAGTGAAGAGAC	TGAAAGCCGATGTGGTCAG
H-Osterix/Sp7	CCAGCAACCCAGAGAAAAG	TTGGCAAGCAGTGGTCTAG
H-ALP	GATGTGGAGTATGAGAGTGACG	GGTCAAGGGTCAGGAGTTC
H-Col1A1	CCCCTGGAAGAATGGAGATG	TCCAACCACTGAAACCTCTG

2.6 Drug Release

2.6.1 FITC-BSA

In order to evaluate the drug release of the fibrin gel system, we utilized a BSA release model. The stock solution was made by dissolving 50mg of FITC-BSA (Sigma-Aldrich,

USA) within 10mM Tris Buffer to make a 10mg/mL solution. The solution was then used in two different methods to evaluate drug release based on pharmaceutical loading literature: soaking in drug solution or cross-linking into the fibrin gel. Gels were either manufactured with or without the FITC-BSA solution to evaluate the release. Final loading was around 300ug/mL within the gel formulation. Soaking was done by placing regular gels within a 300ug/mL FITC-BSA solution for 30 min to allow for absorption. The gels were placed within a 24-well plate and filled with 1mL 1xPBS. At each timepoint the PBS was extracted and evaluated for FITC content through a plate reader at 490nm absorbance. Daily release and cumulative release were fitted into a table to visualize drug release.

2.7 3D Mineralization

2.7.1 uCT of 3D Fibrin Gels

Fibrin gels seeded at 200,000 cells/gel were cultured for 4 weeks within osteogenic supplement media to determine degree of mineralization within the tissue. Following the 4-week culture, the gels were fixed in 10% formalin for 3 hours. The gels were then washed in PBS and placed into 2.0mL microcentrifuge tubes for microCT evaluation. The evaluation was done using a microCT machine (Micro-CT 40, Scanco Medical, Bruttisellen Switzerland) to image the 3D gels. Samples were scanned with a 30uM voxel size, voltage of 45kVp, and current of 200μA. We utilized the ‘Trabecular Bone Analysis’ function through the evaluation tab to process the images. The lower parameter was set to 100 and upper parameter was set to 10000. After contouring the image, we

generated a PDF of the values and compared them across samples. We gathered the values of Bone Volume (BV), Tissue Mineral Deposition (TMD), Tissue Volume (TV), and Total Mineral count.

CHAPTER 3. RESULTS

The objective of this study is to investigate FK506's effect on osteogenesis within MDC and hMSC cell populations and any potential adjuvants. After establishing the objective above, a 3D in vivo model will be constructed to further verify and validate the results. Osteogenesis will be evaluated through biochemical assays, immunostaining, μ CT data, and RT-qPCR.

3.1 Effect of FK506 on Marrow Derived Cells (MDCs) without Osteogenic Medium

During ankle fusion surgery, a microfracture technique is performed on the subchondral bone allowing marrow to permeate the injury site. We thought that by using a marrow population this can simulate the drug's effect more realistically. These experiments serve to determine FK506's effect on this marrow derived cell population and to provide a translatable cell culture model for osteogenesis research. In order to establish a baseline osteogenesis level, the following assays were done without the use of osteogenic differentiation medium.

3.1.1 FK506 Concentration

Since previous work done by Sangadala was performed on C2C12 cells, we wanted to determine the optimal concentration for the MDCs. Alizarin Red S staining was chosen as the evaluation metric for this study. The cultures were evaluated for 14 days in order to verify work done by Sangadala. Concentrations chosen are reflected within the original

paper done with C2C12 cells. It was found that the 1.5 μ M concentration of FK506 had the highest absorbance value compared to the other concentrations and the control across 14 days of culture (**Figure 4**). Other values of 3 μ M and 6 μ M also displayed absorbance values higher than the control.

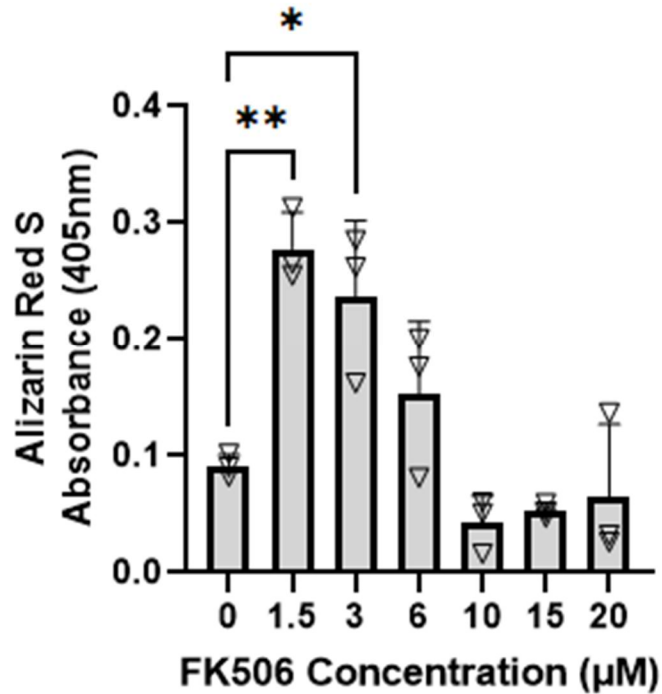


Figure 4. 14- day Alizarin Red Staining absorbance following FK506 treatment within MDCs. Cells standardized with 0.01% DMSO per well. (* p <0.05, ** p <0.01). (n=3)

It was also noted that concentrations higher than 10 μ M exhibited cell lifting within the culture plate. This contributed to the low alizarin staining on the culture plate since there aren't any cells to deposit mineral. Additionally, staining was verified by imaging the monolayer under the ECHO Revolve Microscope. Calcium nodules were identified as

dark maroon spots. From these results, we decided to move forward with the 1.5 μM concentration as our standard dosage for FK506 within the MDCs.

3.1.2 Identification of potential FK506 adjuvants

Once the optimal concentration of FK506 had been established, our area of focus shifted to identifying other molecules that could enhance the FK506 signaling. Since FK506 acts as a secondary BMP signal *in vivo*, it is thought that adding an adjuvant could make FK506 treatment clinically relevant. A literature review was conducted to identify current clinically used molecules that enhance osteogenic signaling. From the search, four osteogenic molecules were purchased to test within our osteogenesis assay: Simvastatin, PDGF-bb, Tamoxifen, and Triiodothyronine.

Simvastatin is a statin class molecule that reduces the amount of cholesterol in the body. The method of activation is by inhibiting HMG-CoA reductase. In a study done by Soo Bae, they discovered that loading a hyaluronic acid (HA) hydrogel with 1mg of simvastatin significantly increased osteopontin (OPN) and alizarin staining levels compared to control in MC3T3-E1 cells [45]. Results were verified within a mouse calvarial defect model. PDGF-bb is a growth factor associated with cell proliferation and several chemotactic/mitogenic effects. PDGF-bb has been extensively shown to increase osteogenic differentiation within *in vitro* and *in vivo* models [32, 33]. Tamoxifen is defined as a selective estrogen receptor modulator. Tamoxifen has been shown to increase bone mass in postmenopausal women [46]. Xie et al found that low dosage tamoxifen injections increased femoral BV/TV and significantly increased osteoblast

markers [47]. Triiodothyronine (T3) is a thyroid hormone that contributes to skeletal development and fracture healing [48]. T3 has been shown to promote osteogenic differentiation in C3H10T1/2 cells and promote AMPK/p38 phosphorylation [49]. Following the literature review, the pharmaceuticals were ordered and aliquoted per the studies listed above.

Within a 24-well plate, the MDCs were treated with the optimal concentration of the osteogenesis molecule in DMEM (2% FBS, 1% PSF). All combinations were tested with other drug and as a standalone treatment. Following a 14-day study, the plates were fixed and evaluated for Alizarin Red S staining. The plates were then imaged using an EPSON scan and absorbance at 405nm was read using a plate reader.

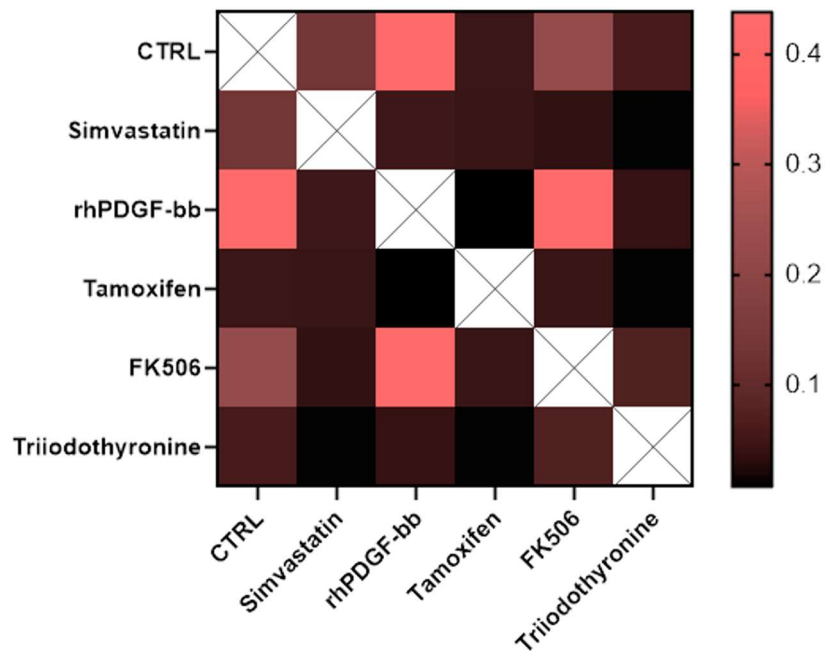


Figure 5. Combinatorial Study: 14-Day Alizarin Red S Staining absorbance at 405nm. Heatmap produced from absorbance values from drug treatments: Simvastatin (1 μ M), Tamoxifen (5 μ M), Triiodothyronine (0.1 μ M), FK506 (3 μ M). (n=3).

The heatmap displayed above shows the absorbance values for every combination of drug treatment tested on the MDCs (**Figure 5**). The highest absorbance value was 0.433 for 10ng/mL treatment with rhPDGF-bb. The second highest value was 0.438 for the 3 μ M FK506/PDGF-bb combination. The 3 μ M FK506 group had a value of 0.217. These results suggest that PDGF-bb treatment enhances FK506-induced osteogenesis. Additionally, PDGF-bb could be used as a stand-alone molecule. The other ‘osteogenic’ molecules that had been identified using the literature results did not exhibit the same levels of osteogenesis seen from PDGF-bb. These results were re-verified in a similar study testing compounds with an increased absorbance from the control (simvastatin, rhPDGF-bb, tamoxifen). The re-verification confirmed rhPDGF-bb as the leading candidate for osteogenesis. From here, further investigation is needed to verify these results in other osteogenic assays (ALP, RT-qPCR, etc). Additionally, concentrations of rhPDGF-bb were tested with FK506 to determine the optimal combination treatment and the 10ng/mL dosage had the highest mineral deposition.

3.1.3 ALP Assay for FK506 and PDGF-bb

To verify the results from the combination assay, the treatments will be applied to the bovine MDCs and evaluated through an alkaline phosphatase assay. ALP levels typically measure the differentiation of mesenchymal stem cells (MSC’s) to osteoblasts. For this study, this assay measures intracellular ALP levels following drug treatment. MDCs were cultured in a 24 well plate and treated for 14 days in DMEM (2% FBS, 1% PSF). Following the 14-day culture, the plate was analysed for ALP activity. Cells were lysed and the supernatant was collected. The results of this study show that the combination of

FK506 and PDGF-bb significantly increase the intracellular ALP levels compared to the singular treatment and control treatment groups (**Figure 6**). PDGF-bb exhibited low ALP values, contrasting from the alizarin red s absorbance values earlier. Since PDGF-bb is a known proliferative factor, standardizing to the protein count (Bio-Rad, BSA) makes the metric on a per cell basis. With the alizarin s stain, it cannot be normalized to protein count. This result produces the question of if PDGF-bb initiates a low level of osteogenesis, but the number of cells creates a high signal.

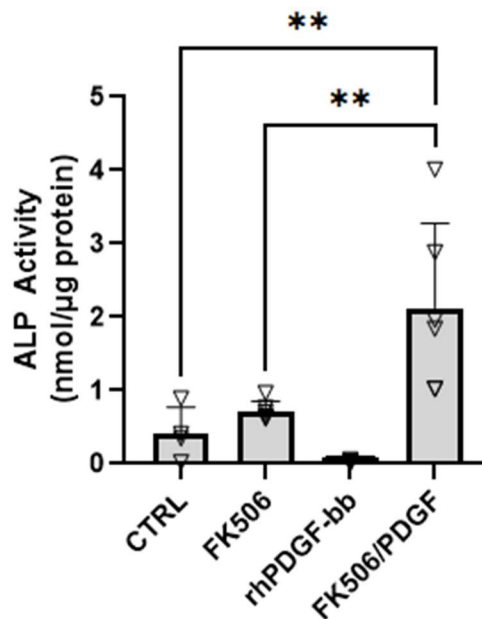


Figure 6. 14-Day Alkaline Phosphatase Activity of MDCs without Osteogenic Medium. Cells treated with 1.5 μ M FK506 and 10ng/mL rhPDGF-bb. Activity was standardized using total protein amount (Bio-Rad BSA kit). (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). (n=6)

The ALP Assays are typically done at 3-7 days from the first osteogenic stimulus since ALP is one of the earliest markers for osteogenic differentiation. When doing the 24-well

cultures it was hard to obtain noticeable ALP expression for 7 days, so the culture was extended to 14 days in order to reach a difference. Later studies are done within a 6-well plate.

3.2 Effect of FK506 on MDCs within Osteogenic Medium

After establishing a baseline level of osteogenesis within the first few studies. We wanted to evaluate the above treatments within osteogenic medium. By evaluating cells within an active osteogenic environment, we can better predict the translational value of our pharmacological treatments. Our lab decided to first use an osteogenic differentiation supplement (Stem Cell Technologies) to perform preliminary studies while osteogenic media materials were being shipped/ordered. The following studies will be labeled as using the osteogenic supplement or lab-made OM media.

3.2.1 Effect of FK506 and rhPDGF-bb in Osteogenic Medium

Within a 24-well plate, 25k bovine MDC cells were plated onto the well and fed with either control media (2% FBS DMEM), 1.5uM FK506/PDGF-bb media (with supplement OM or in 2% FBS), or OM control media. The plate was evaluated after 14 days of culture to determine mineralization. During the culture, it was noticed that around day 10 that cell spheroids started to form. Spheroids formed within almost every group that contained the OM supplement. It was hypothesized that since the supplement contains FBS and growth factors, the abundance of cells caused them to fold in and clump together. Due to the nature of the alizarin red s stain, any well with spheroids cannot be evaluated fully. However, the staining within the control OM groups revealed robust

mineralization not seen within the basal media groups. This further pushed us to investigate the effect of our drug within OM.

This study was repeated using a lower cell count (10k cells per well) and evaluated on Day 7 and Day 14 for Alizarin Red S absorbance. Wells were fed with 1.5uM FK506, 10ng/mL PDGF-bb, and the combination for both sets (n=6). The results were imaged (EPSON Scanner) and absorbance read using a plate reader. The 7-day results show that the treatment with 1.5uM FK506 alone produced the highest absorbance values compared to the control and PDGF-bb containing wells (**Figure 7B**). Within day 14, absorbance values from FK506 and PDGF were higher than the control and were similar to each other. Upon closer look of the wells using the ECHO Revolve Microscope (Brightfield Setting), the difference in mineralization is more apparent between the control and treatment groups. Another observation is the presence of dark nodules within the FK506-PDGF combination group, where this could indicate matured mineral. This adds another factor into our analysis of the alizarin-stained plate.

Once again, the FK506 and PDGF-bb combination group exhibited spheroid formation even with the lower cell count (**Figure 7a**). Since spheroid formation seemed inevitable, we decided to focus on assays that were unaffected by this phenomenon (ALP, rt-qPCR). We decided to look at the ALP assay done with the MDCs in basal media and re-visit it within osteogenic media. We wanted to see if the drug treatments had any synergistic effect with the osteogenic media and to determine if there were any differences between the two runs. D35 of the MDC cell population was used for this experiment. Following two weeks of culture, the cells were harvested and evaluated for intracellular ALP. From

the results, there was a significant increase in ALP activity across all treatment groups compared to the control. FK-PDGF being the highest out of the groups (**Figure 7C**). There is also a significant difference in ALP activity from FK506 compared to the control. rhPDGF-bb also shows an increase compared to the control. This is different from the results of the original assay in basal media (**Figure 4**).

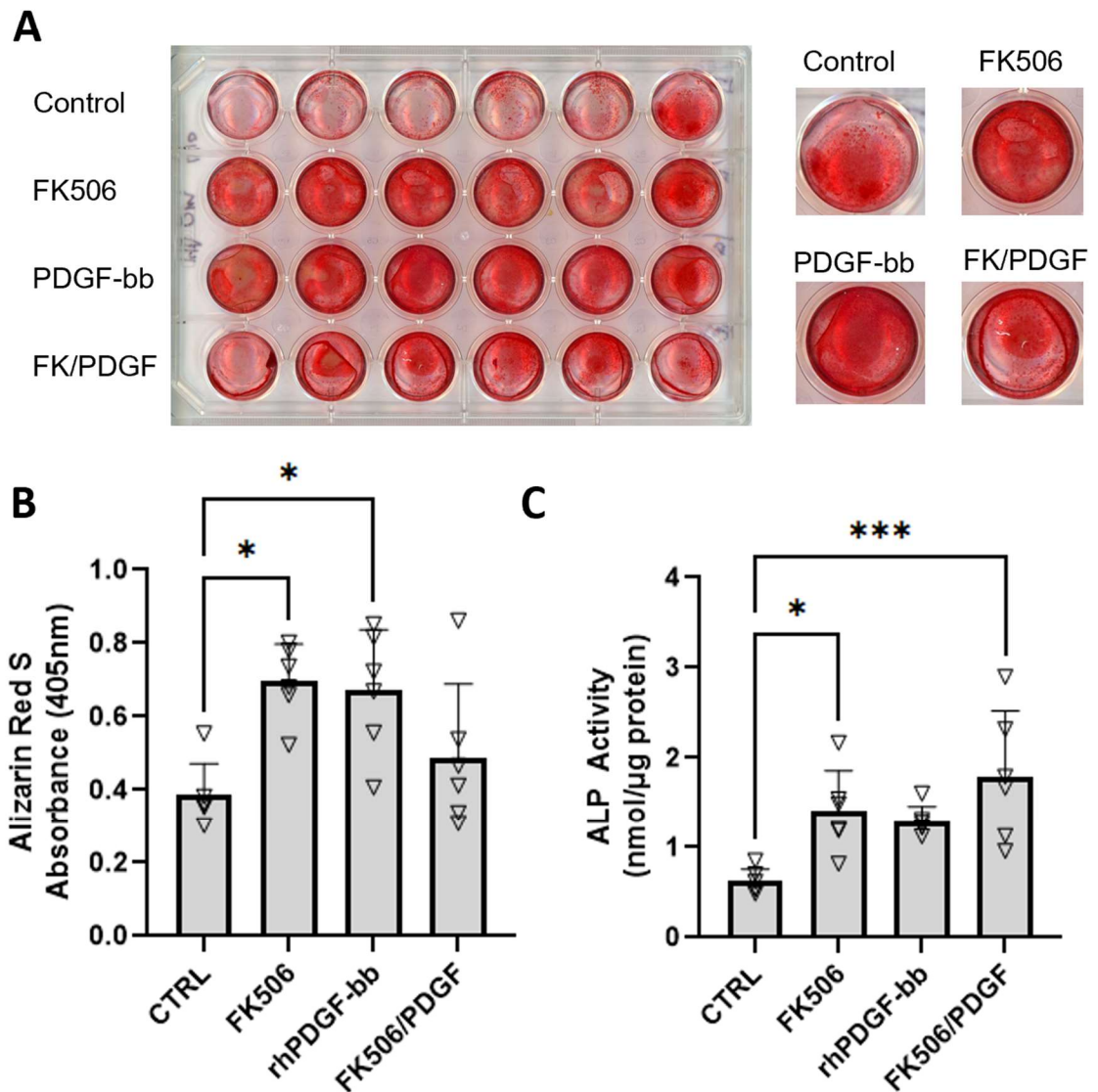


Figure 7. 14-day Alizarin Red S staining and ALP activity of MDCs in Osteogenic Medium. **A:** Photo of 14-day Alizarin Red staining in A. Best case sample visualized on the right. (EPSON Scanner). **B:** Alizarin Red stain of D10 MDCs following 14 days culture (n=6). **C:** 14-day ALP Activity of D35 MDCs treated with 1.5 μ M FK506 and 10ng/mL rhPDGF-bb (n=6). Activity standardized using total protein amount (Bio-Rad BSA Kit). (EPSON Scanner). (*p<0.05, **p<0.01).

3.2.2 *Temporal Activation of Osteogenesis*

Adding onto the question posed by the previous alizarin stain, the maturation of mineralization proposes a temporal aspect to our overall analysis. Regarding the differentiation process, there are stages where the osteoprogenitor cell becomes a preosteoblast and eventually an osteoblast. Our focus has shifted to what FK506 and PDGF-bb are doing at early and late timepoints to ‘push’ the cell to an osteogenic phenotype. One of the ways that we can look into this is through rt-qPCR. An array of early and late genes that signal for osteogenesis was obtained and evaluated at 3 and 14-day timepoints. These timepoints were chosen based on previous literature concerning osteoblast differentiation.

3.2.2.1 Temporal Aspects and Donor Variability

Bovine MDC cells were analyzed across two different time points: 3 days and 14 days to determine temporal effects of the treatments. Different bovine donors were assessed to investigate any variability between cell samples.

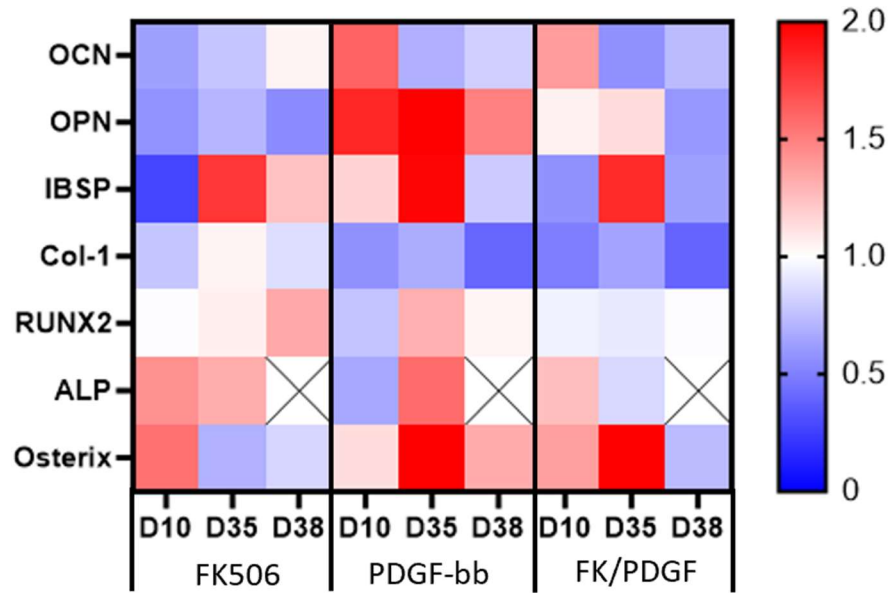
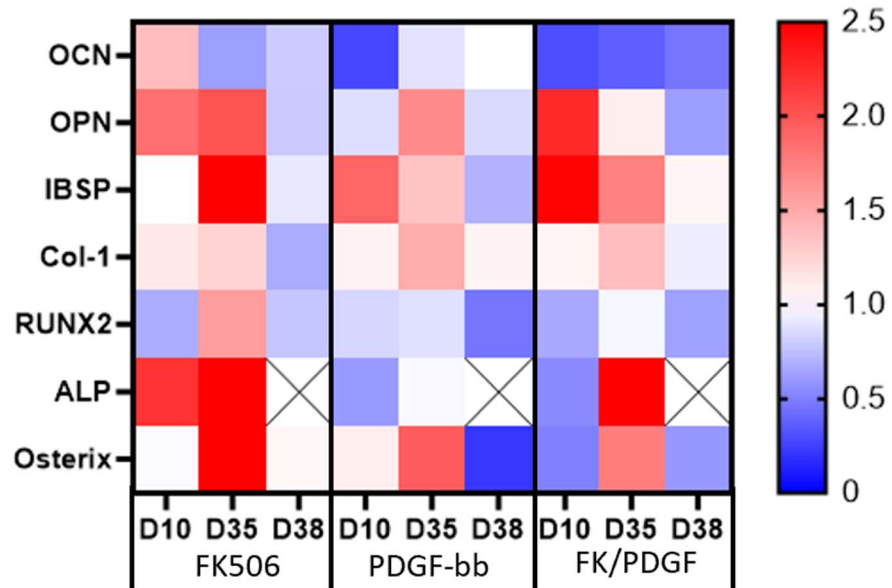
A**72 Hour Gene Expression****B****14-Day Gene Expression**

Figure 8. 72 Hour and 14-Day rt-qPCR expression of MDCs in Osteogenic Medium. **A:** 72 Hour rt-qPCR expression (n=3). **B:** 14-day rt-qPCR expression (n=6). Samples unable to read are expressed as 'X'.

Following 3 days of culture within the osteogenic media, FK506 had increased gene expression for ALP and Osterix (Sp7) genes (**Figure 8A**). PDGF had an increase within Osterix, IBSP, OPN, and OCN compared to control. The combination had an increase within ALP, Osterix, and OCN. For the 14-day culture, there were some interesting differences between the two time points. FK506 showed increases in IBSP, Osterix, Col-1, OPN, and Runx2 compared to the control (**Figure 8B**). PDGF-bb showed increases in Col-1 and OPN. The combination showed increases in IBSP, Osterix, and Col-1. This is widely different from the 3-day results as FK506 showed significant increases across 4 osteogenic genes.

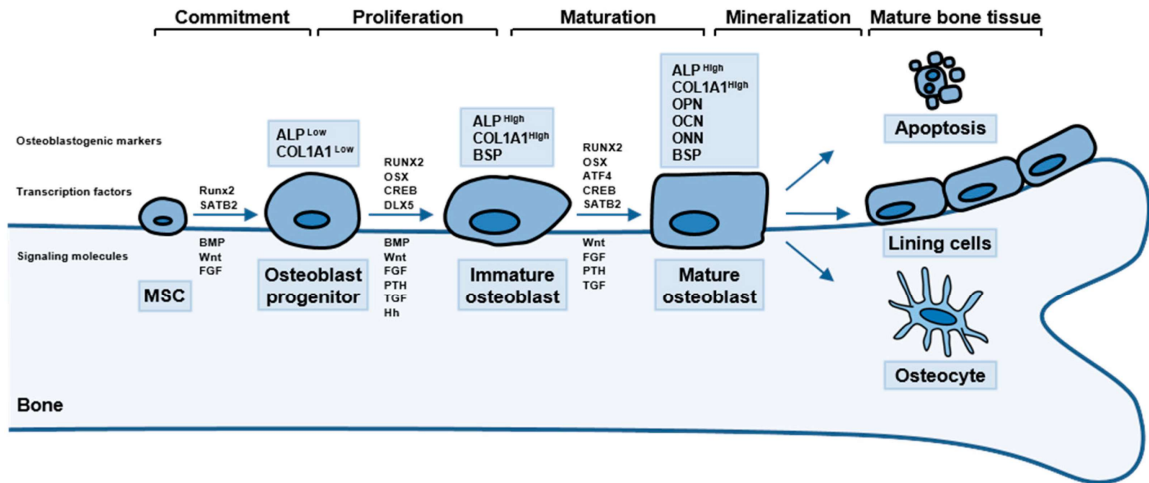


Figure 9. Osteoblast differentiation pathway for mesenchymal stem cells. Adapted with permission [50] via Creative Commons Attributions License. Copyright © 2021, International Journal of Molecular Sciences.

From **(Figure 9)** we can attempt to map out where the cells are within the differentiation stage. Another aspect of this is determining what treatments are affecting which genes at the timepoints. If we look into our D10 donor, we can see that at 3 days of treatment there are differences between each treatment group. The FK506 group expresses ALP and Osterix, where PDGF-bb is expressing OCN, OPN, IBSP, and Osterix. This observation changes at 14 days where FK506 expresses OCN, OPN, IBSP, Col-1, and ALP. Compared to PDGF-bb expressing IBSP and Col-1. From this data, it seems that FK506 differentiates the cell into a matured osteoblast at 14 days compared to the PDGF-bb treated group. This opens up another question into whether PDGF-bb can help early activation where FK506 can help late activation of osteoblasts.

These results were compared with the D35 donor and some similar observations were observed. FK506 treatment had expression for IBSP, Col-1, Runx2, and ALP. This falls within the immature osteoblast phenotype at 3 days. At 14 days, the FK506 treatment had increases in all genes except OCN pushing to a mature osteoblast phenotype. The PDGF-bb and FK/PDGF groups had similar genes upregulated/downregulated however, for 14 days both Runx2 and ALP were downregulated. FK/PDGF exhibited a similar genotype compared to the PDGF-bb treated group within the D35 group.

For the final donor we decided to test out the D38 donor for 3 and 14-day expression. From the gene expression data, we observed that there were not as many upregulated genes compared to the other MDC donors for 3 days. This is especially reflected within the 14-day data where there was not an increase noted for many treatment groups. Additionally, many of the genes could not be read from low CT values. This posed a lot

of questions about the osteogenic potential of this donor and we decided to perform an alizarin red s stain and an ALP assay to verify. From these results we found that there was not a significant increase in ALP content compared to the control. The alizarin stain revealed little mineralization of the cells. Comparing the D38 donor with the D10 and D35, it is apparent that there are some major differences between the MDC donor populations. The D38 donor could be characterized as ‘non-responsive’ to the osteogenic treatment and is a stark difference between the positive results of the D35 and D10 donors. Once again, this adds another layer to our discussion and analysis.

Across all treatment groups, it was hypothesized that the FK/PDGF treatment would overlap expression of FK506 only and PDGF-bb only treated groups. What was thought as the best combination from the alizarin red s and ALP staining did not produce significant results compared to the single treatment groups. Most of the gene expression from FK/PDGF mirrored the expression from PDGF-bb only and only had a few differences within the MDC population. This observation was different than expected and prompted us to re-evaluate our initial hypothesis. We can also investigate our evaluation methods as Alizarin staining and ALP are both cumulative tests while rt-qPCR is a single snapshot of the cellular activity. Though this does provide valuable information into the specific pathways that can be affected by treatment, this does question the efficacy and accuracy of the metric. Thus, it is important to have a holistic approach to evaluating the proposed treatment.

3.2.2.2 Does FBS concentration affect osteogenic assays?

Fetal Bovine Serum (FBS) is a common reagent used within cell growth media. FBS contains growth factors, hormones, proteins, etc that are isolated from drawn blood. Since FBS is isolated from an individual sample, there can be variability between different lots. This can include different protein concentrations and other constituents that can affect cell growth [51]. FBS also contains ALP, which can affect mineralization of osteoblastic tissue. Additionally, our earlier studies had been made with 2% FBS media and longer experiments can run the risk of serum starving the cells. After consulting with Dr. Sangadala we decided to test osteogenic differences between high FBS (10%) and low FBS (2%) between treatment groups.

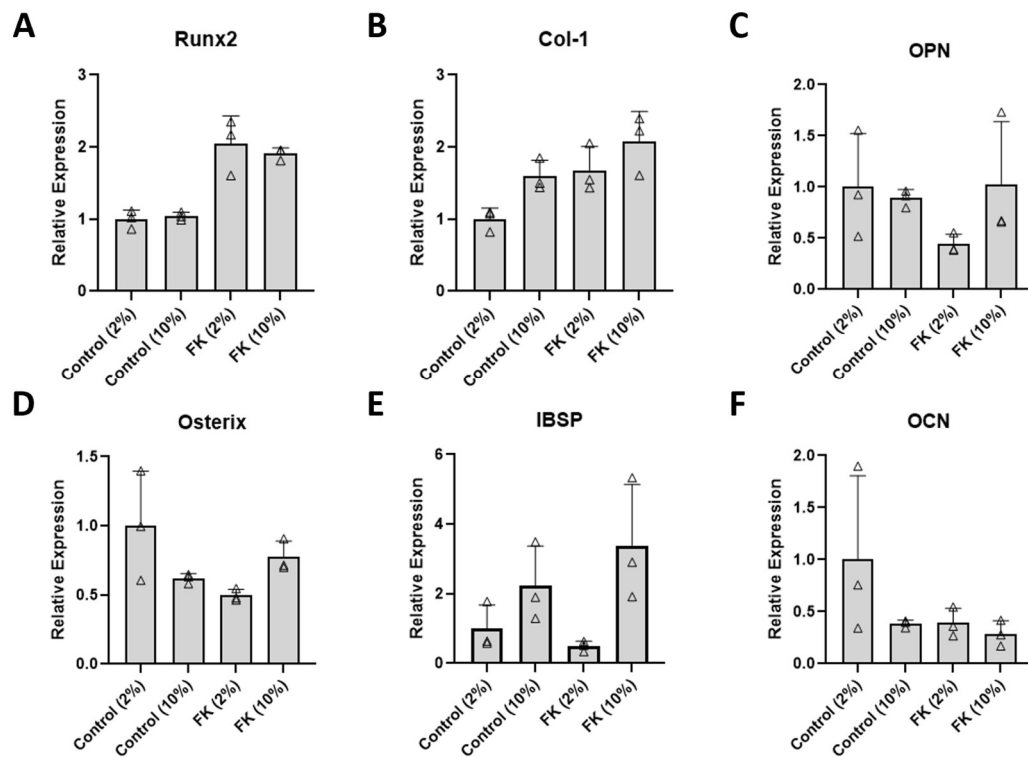


Figure 10. 7-Day rt-qPCR expression for D10 MDCs. A-F: Gene Expression for RUNX2, COL1A1, OPN, Osterix, IBSP, OCN.

MDCs were plated in osteogenic media for 7 days and analyzed for rt-qPCR. We ran the plate with high/low FBS and with/without FK506. From the results it was observed that Runx2 was increased within the FK506 containing groups regardless of FBS concentration. However, genes such as IBSP and Col-1 had an increase in gene expression compared to low FBS (**Figure 10**). Osterix and OCN showed decreased gene expression within the high FBS. The IBSP expression is intriguing to look at for the FBS concentration differences. Between 2% and 10% concentration, there is a significant difference in expression in each treatment group.

3.2.3 Runx2 Localization of MDCs

Another method of analysis that we can employ is evaluating nuclear localization of differentiating factors. For our purposes we decided to investigate Runx2, the master osteoblast differentiation gene. By staining for Runx2 within the cell, we can determine the responsiveness to pharmacological treatment. This data also gives us information on a cell-to-cell basis. This is especially important within such a heterogenous cell type such as the MDCs to be able to identify responding and non-responding cells. The evaluation is based off of how much staining resides within the nucleus versus the cytoplasm. The ratio of Nucleus to Cytoplasm staining is calculated per cell to determine how responsive Runx2 expression is to the treatment. This data can be organized into clusters to determine mean averages and potentially active cells. Evaluating the population as a whole can centralize our average and make a significant difference seem actually

insignificant. This can be combined with future flow cytometry data to identify which populations of MDCs have a higher proportion of osteoblasts/MSCs to evaluate healing potential. For our purposes, we used the entire population of the cells without sorting into clusters to get an initial look at this metric.

For this experiment, cells were given control OM supplement, 1.5uM FK506, 10ng/mL PDGF-bb, and FK/PDGF media. The cells were evaluated after 3 days of culture and stained for Runx2 expression. From the results we can see that there is a significant increase in responsiveness within the FK506 containing groups (**Figure 11**). In both basal media and osteogenic media, the FK506 containing group had a significantly higher intensity stain. The basal media FK506 group and OM control groups are not significantly different, suggesting that FK506 provides the same stimulus as OM. Since this is an entire population subset, the variation and range in data can be quite high. It is important to look at the mean of the data sets where we can get significant differences between entire population subsets. Further data needs to be collected to make a correct determination between treatment groups.

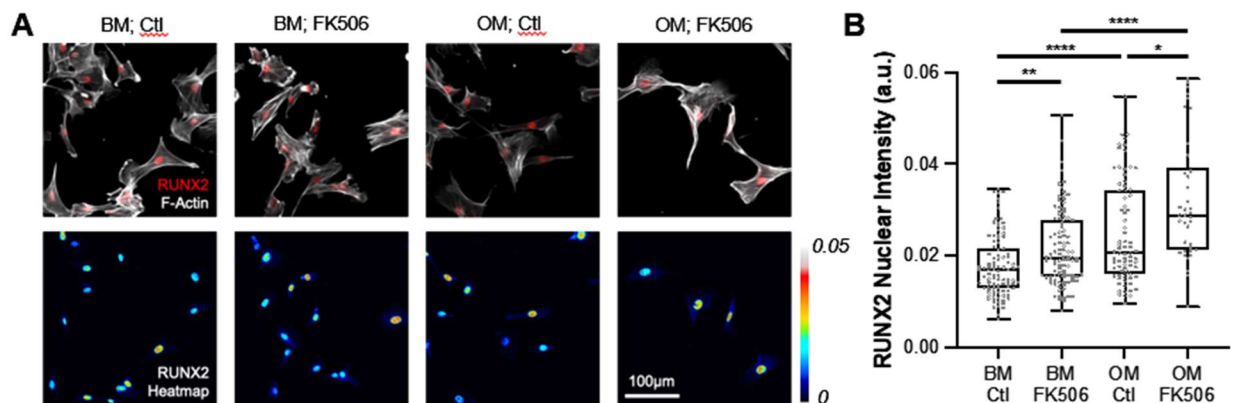


Figure 11. Nuclear Localization of RUNX2 staining on MDC cells. **A:** Split channels for RUNX2 (red) and F-actin (gray) and heatmap of relative intensity below. **B:** Nuclear intensity of RUNX2 staining showed an increase in localization when treated with 1.5 μ M FK506. Statistical significance was determined using one-way ANOVA (**** $p < 0.0001$, ** $p < 0.01$, * $p < 0.1$).

3.3 Effect of FK506 on Human Mesenchymal Stem Cells (MSCs) in Osteogenic Medium

One of the problems with using a heterogenous cell type such as the MDCs are that individual cell assays (such as the nuclear localization) may not give observable differences within osteogenesis. Additionally, for our research we want to focus on FK506's effect on bone forming cells such as osteoblasts. One of the key components of the healing cascade is the migration of cells into the wound site to heal the area. Stem cell migration helps to provide new osteoblasts and osteoclasts to remodel and change the surrounding tissue. By choosing bone marrow derived hMSC's we can accurately model how our treatment affects these migrating cells. Using stem cells also extends the differentiation timeline that we can observe, allowing us to better grasp how our treatment affects the different stages.

3.3.1 hMSC Mineralization and Dose Dependence

Once we had our new cell type, we wanted to determine the osteogenic potential of the hMSCs. We decided to perform an alizarin stain and an ALP assay on a monolayer of cells. First, we wanted to observe how the hMSCs respond to the FK506 treatment and the osteogenic supplement. We set up a plate monolayer of different FK506

concentrations (0.5uM, 1.5uM, and 3uM) along with either a 1:4 or 1:10 dilution of OM supplement. The plate was cultured for 14 days, and mineral content was evaluated at the end using Alizarin Red S. In Figure 12 we observed that the 1:4 dilution produced the highest alizarin absorbance and the largest difference between the FK506 groups and the control compared to the 1:10 dilution. However, there were no differences between FK506 concentrations in the 1:4 dilution. This contrasts the 1:10 dilution where the 1.5uM FK506 concentration had the highest absorbance value.

From here we decided to move forward with the 1:4 dilution and the 1.5uM FK506 concentration. All treatment groups were then checked within an alizarin stain for 14 days. It was noticed that after 10-12 days, dark nodules started to form within the wells (**Figure 12A**). This was observed within the ECHO microscope following the staining. It is believed that this is mature mineral. Following the absorbance calculations, the PDGF-bb and FK/PDGF-bb groups produced the highest mineral following the 14-day assay.

Finally, we checked this within an ALP assay. The ALP assay was done within a 6-well plate seeded with 100,000 cells per well. This was evaluated for 3 days to determine early ALP production. From the results, the FK506/PDGF and PDGF-bb treatment group produced a significantly different value compared to the control (**Figure 12C**). The PDGF-bb group had the highest value (1.87 nmol) versus the control (1.42 nmol). FK506 had a lower ALP value than the control, however this was recovered within the combination group of FK506/PDGF-bb. Basal media was used as a negative control to investigate the effect of FBS concentration on our study. Since the basal media group was

lower than the OM group, we can identify that FBS does not drastically affect our ALP Assay.

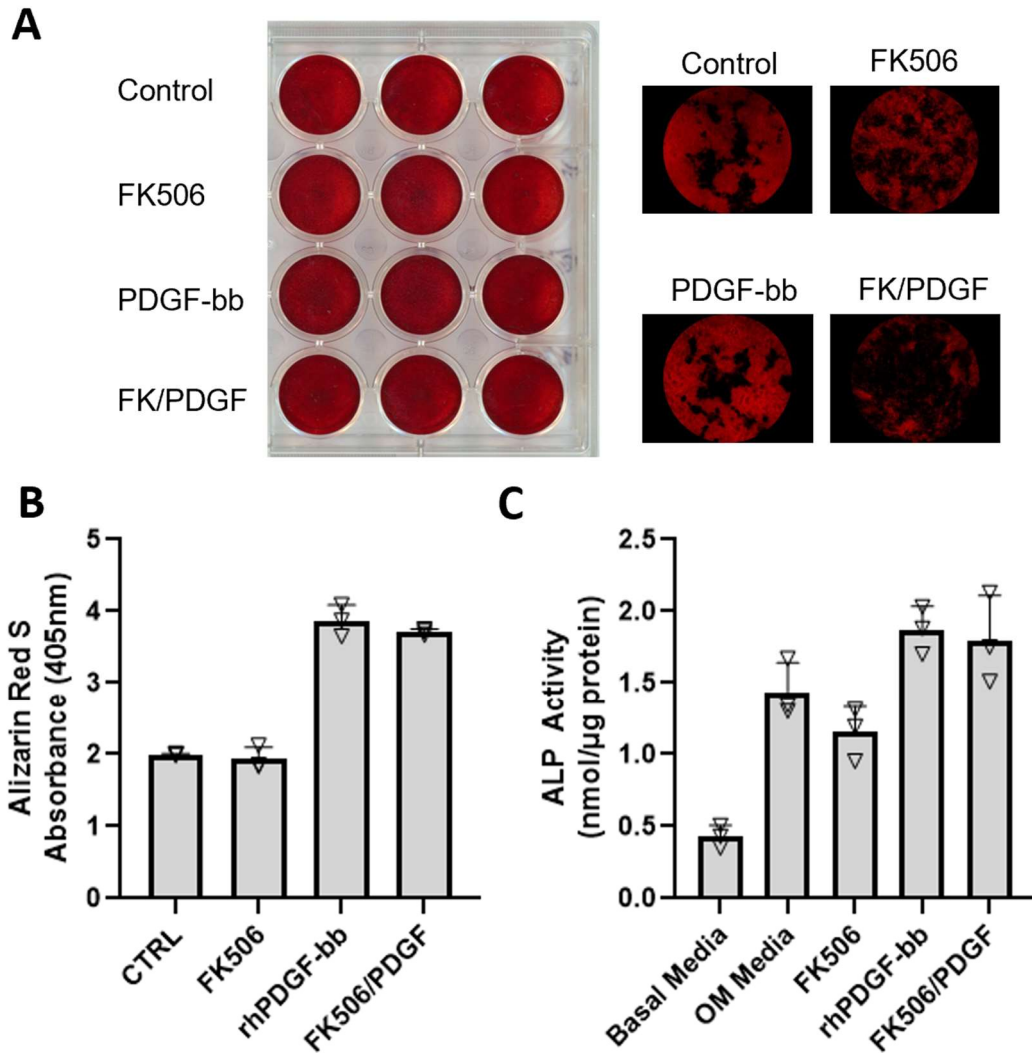


Figure 12. 14-day Alizarin Red S staining and ALP activity of hMSCs in Osteogenic Medium. **A:** Photo of 14-day Alizarin Red staining in A (EPSON Scanner). Best case scenario visualized to the right (ECHO Revolve). **B:** Alizarin Red stain of hMSCS following 14 days culture (n=3). **C:** 14-day ALP Activity of hMSCs treated with 1.5μM FK506 and 10ng/mL rhPDGF-bb (n=3). Activity standardized using total protein amount (Bio-Rad BSA Kit).

3.3.2 Temporal aspects of hMSC cells

As with the MDCs, we wanted to evaluate the differentiation process across early (3 day) and late differentiation stages (14 days). Early differentiation yielded different results from the MDCs. Runx2, Col-1, and OCN were all upregulated for FK506 compared to the control. FK-PDGF saw an increase within Col-1, however that was the only upregulated gene (**Figure 13A**). All other genes were downregulated for the treatments compared to the control. For the 14-day expression, Col-1 saw a slight increase for FK506 along with Runx2 (**Figure 13B**). All other genes were downregulated.

From the results it appeared that the treatment did not result in the same expression that we saw with the MDCs earlier.

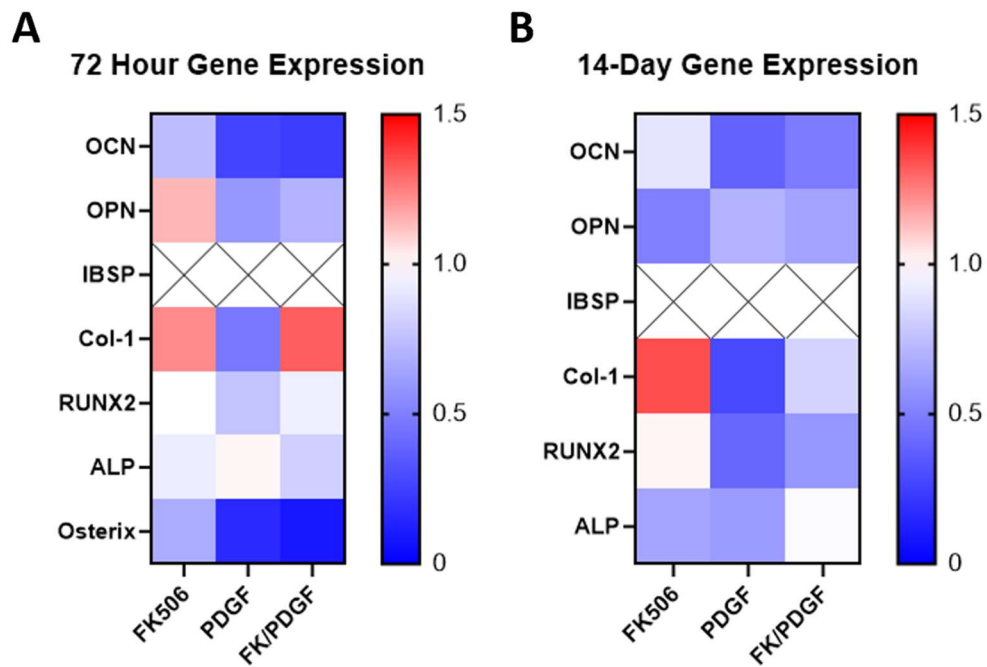


Figure 13. 72 Hour and 14-Day rt-qPCR expression of hMSCs in Osteogenic Medium. A: 72 Hour rt-qPCR expression (n=3). B: 14-day rt-qPCR expression (n=3).

3.4 In Vitro 3D Gels

In order to better understand and model the fusion environment we decided to use a 3D culture model. Our lab had been using fibrin gel gels as a culture system for chondrogenesis, so it was easy to translate to our work. One of the strengths of using the fibrin system is that the system is tunable and easy to integrate. However, one of the struggles with using fibrin is that the gel is very soft in comparison to the hard polystyrene of the culture plate. Since it is known that natural osteogenesis can occur on hard substrates, the fibrin system will not have that advantage. The fibrin gel system does allow for us to look at cell matrix deposition in 3D. This can add a spatial aspect to our ultimate analysis. Gel media will be made with 1:1000 aprotinin. Aprotinin is a protease inhibitor that prevents the breakdown of fibrin so that the gel can be analyzed after the time period.

3.4.1 Matrix Deposition for MDCs in 3D (4 weeks)

The fibrin gels were loaded with 200k cells per 100uL gel. The gels were then cultured in a 24-well plate for 28 days before being analyzed for Alizarin Red staining. The media was made using a 1:4 ratio of supplement OM to basal media along with the addition of 1:1000 of aprotinin. Following 28 days of culture, the gels are fixed and then sectioned.

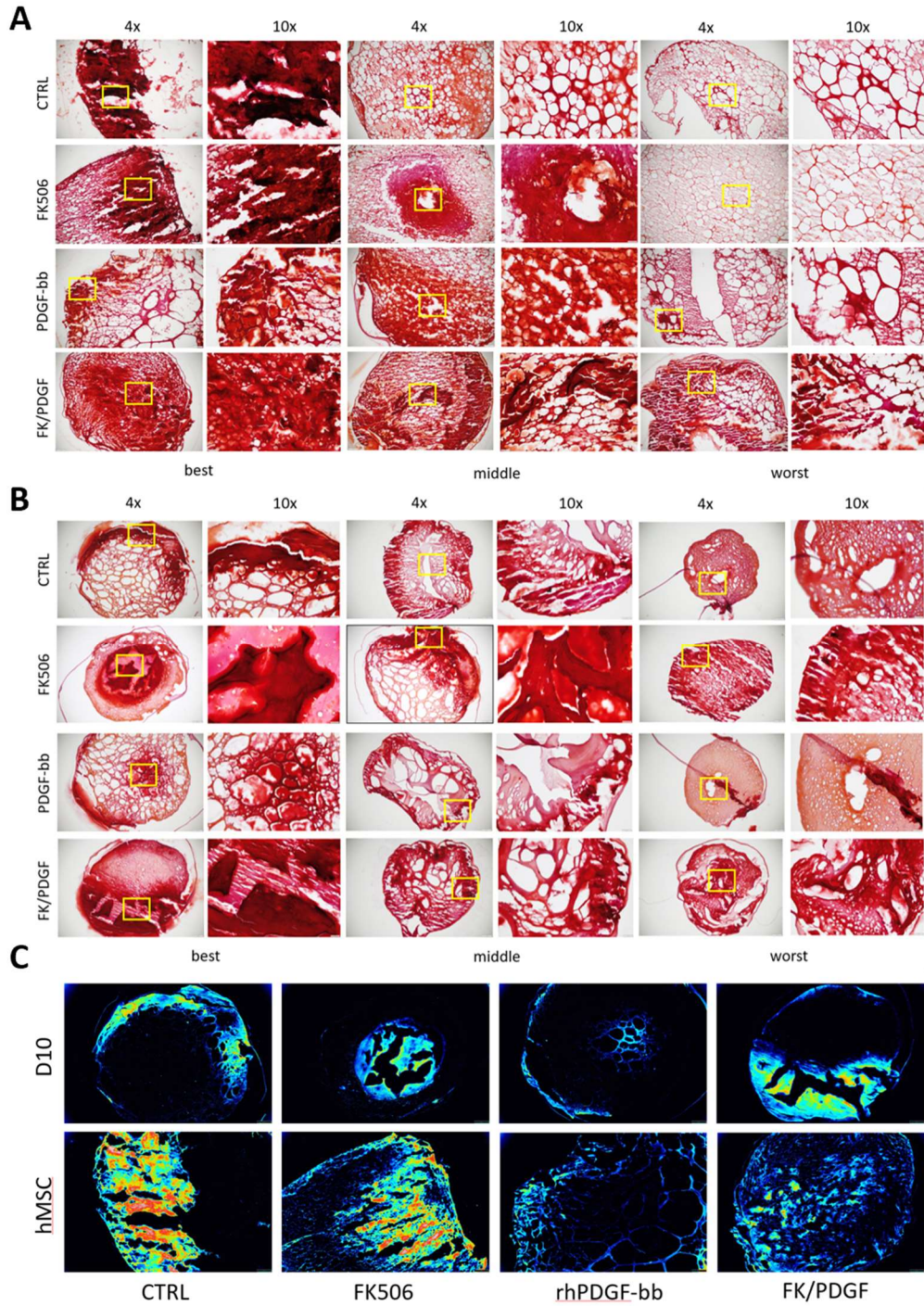


Figure 14. Alizarin Red Staining of 4-week gel cultures and heatmap visualization. Staining ordered from best/middle/worst section observed. **A:** hMSC gel staining. **B:** D10 gel staining. **C:** Heatmap visualization of the ‘best’ images above performed in ImageJ with ‘Royal’ Table Lookup.

We wanted to tackle the variability in the data head-on. We thought that organizing the alizarin stained imaged into ‘best’, ‘middle’, and ‘worst’ categories based on micro-CT data would provide a comprehensive review (**Figure 14A-B**). Essentially, we took the fibrin gels and ranked them on how much bone volume (ccm) that had formed over the 4 weeks. We then chose the highest, middle, and worst bone volume scores and chose those gels to section for the staining. Once we had around 6-8 sections (20 μ M) for each group we stained for alizarin red and imaged the one with the most visualized mineral. Those images were then compiled into the following figure. It is worth noting that each stain is variable due to cell migration and/or location within the initial gel. However, with our method we can still see some striking differences between the groups. For figure C, we generated a heatmap after noticing that there were ‘darker’ regions of the alizarin stain. We decided to visualize this with the ‘best’ case scenarios for gel staining.

The MDC gel staining revealed some interesting findings: the periphery seemed to have a more intense stain, and the mineral deposition was not homogenous throughout the gel (**Figure 14B**). The FK506/PDGF-bb gel had the most intense alizarin stain according to visual observation, while PDGF-bb did not produce an intense stain. The FK506 group seems to have more of these ‘nodules’ than the control, which suggests higher mineral deposition over the two weeks. This is more apparent when you look at the generated heatmaps from within Figure 14C. The red/yellow regions of the heatmaps suggest more mineral. However, an interesting observation is the lack of mineral within the rhPDGF-bb group. This effect seems to be recovered within the FK/PDGF group.

The hMSC gels had the same general trends as the MDC gels. There appeared to be more cells around the periphery of the gel, and this resulted in a more intense alizarin stain. The CTRL and FK506 images reveal an intense stain similar to what was observed within the hMSC monolayer (**Figure 14A**). This is verified within the heatmap to show that the staining we see is a darker red stain than in the original gel. The rhPDGF-bb treated gel did not show intense mineral staining compared to the FK506 gel. This is similar to the FK/PDGF group that follows. Though it does seem that mineral is diffused throughout the FK/PDGF gel, but at a low pixel value. Overall, these differences will need to be verified within the micro-CT section for a cumulative analysis. Though it is nice to look at the histology, the inherent variability within the stain doesn't provide as much confidence as with the micro-CT data.

3.4.2 μ CT Data for Gels

While looking at the mineral staining of the cryosections is nice, the sections cannot fully represent the entire deposition within the gel. We decided to use micro-CT as a metric for evaluating whole bone formation within the MDC and hMSC gels. These gels were cultured out for 4 weeks to ensure robust mineral formation would occur. For these gels, we decided to decrease our FK506 dosage from 1.5 μ M to 0.5 μ M concentration. This was because we were concerned that long-term FK506 administration at higher concentrations could be cytotoxic to the cells. Our hypothesis was that our treatment would result in significant bone formation compared to the control. Following the 4-week culture period, the gels were fixed in formalin and scanned using the micro-CT machine within our lab (SCANCO). The gels were then scanned and evaluated for Bone Volume (BV), Total

Mineral Density (TMD), Tissue Volume (TV), and Total Mineral count. 3D visualizations of the bone area were compiled using the Ray Tracing feature of the SCANCO machine (Figure 14 A-D).

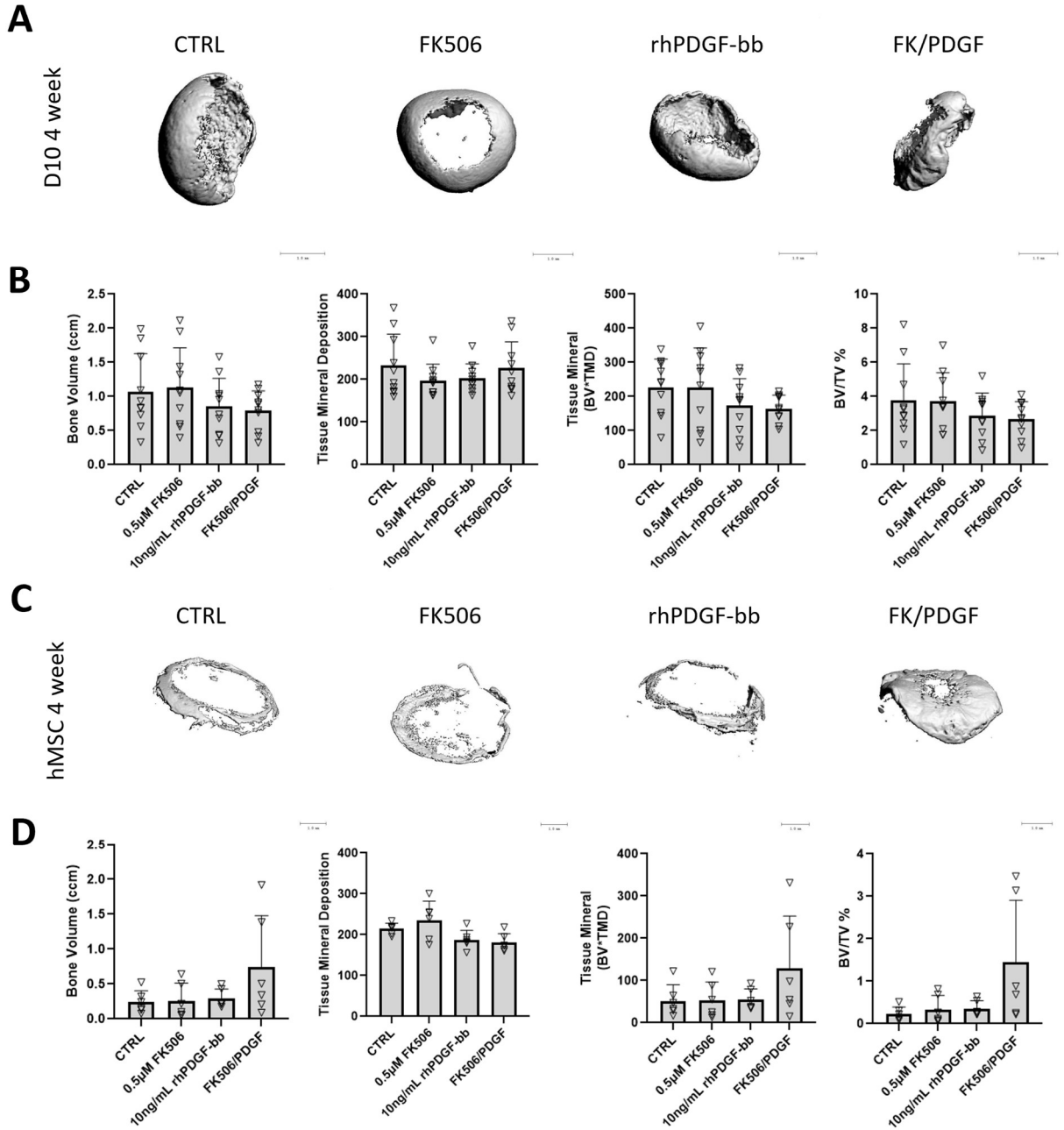


Figure 15. Micro-CT of Fibrin gels cultured for 4 weeks in Osteogenic Medium. A: D10 MDC gels cultured for 4 weeks. **B:** BV, TMD, Tissue Mineral, and BV/TV calculations (n=10). **C:** hMSC gels cultured for 4 weeks. **D:** BV, TMD, Tissue Mineral, and BV/TV calculations (n=6).

From the MDC gels, there was an increase in BV from the FK506 treated group to the control along with the total mineral count. The PDGF and FK/PDGF groups were very similar to each other in terms of BV and total mineral (**Figure 14B**). There was not a significant difference between the two groups. Just like the cryosectioned samples, most of the mineral appeared to be around the edge of the gel when visualized with the Ray Tracer compiler. However, it was noticed that some of the FK506 groups showed mineralization that extended into the gel compared to around the outside. This suggests a more homogenous mineralization. Another parameter to look at is the TMD. This is the average pixel intensity from the micro-CT scan that calculates the amount of 'bone' selected. The average TMD for the MDC gels are around 220 mg HA/ccm while the average TMD for trabecular bone is 450 mg HA/ccm. While it is important to show that there is more mineral within the treatment group, it still falls shy of the average value for trabecular bone.

From the hMSC gels, there were a couple key differences between this and the MDC data. The BV in all treatment groups is higher than the control, with the FK/PDGF group being significantly higher than the other groups (**Figure 14D**). The BV is 0.740 ccm compared to 0.24 ccm in the control. The TMD is highest in the FK506 group with 235mg HA/ccm compared to 214.4 mg HA/ccm control. The total mineral is 128 mg HA in FK/PDGF compared to 51mg HA in the control. Similar to the MDC group, most of

the mineral was around the periphery of the gel (**Figure 14C**). Compared to the BV and TMD values of the MDC gel, the values of the hMSC gels were much lower. The average BV of the control within the MDC group was 1.02mg HA versus 0.24mg HA in the hMSC group.

3.5 Future Directions

3.5.1 Drug Release

We have an idea of what treatment we want to deliver for our in vivo model, but one of the most intriguing questions regards the delivery of the pharmaceutical. Most papers typically just administer via systemic infusion, which is easy to do but can result in additional problems. Additionally, the type of release can drastically change the clinical effectiveness. If we think back to BMP-2, one of the main issues was the high dosage within the sponge. This was soaked onto a collagen sponge and follows a burst release profile. For our lab, we wanted to use a carrier scaffold to deliver the FK506 and decided to use the fibrin gel from the ease of access. We tested two different manufacturing methods for the drug release: crosslinked and soaked. Release data is shown below for the cured and soaked scaffolds (**Figure 16**).

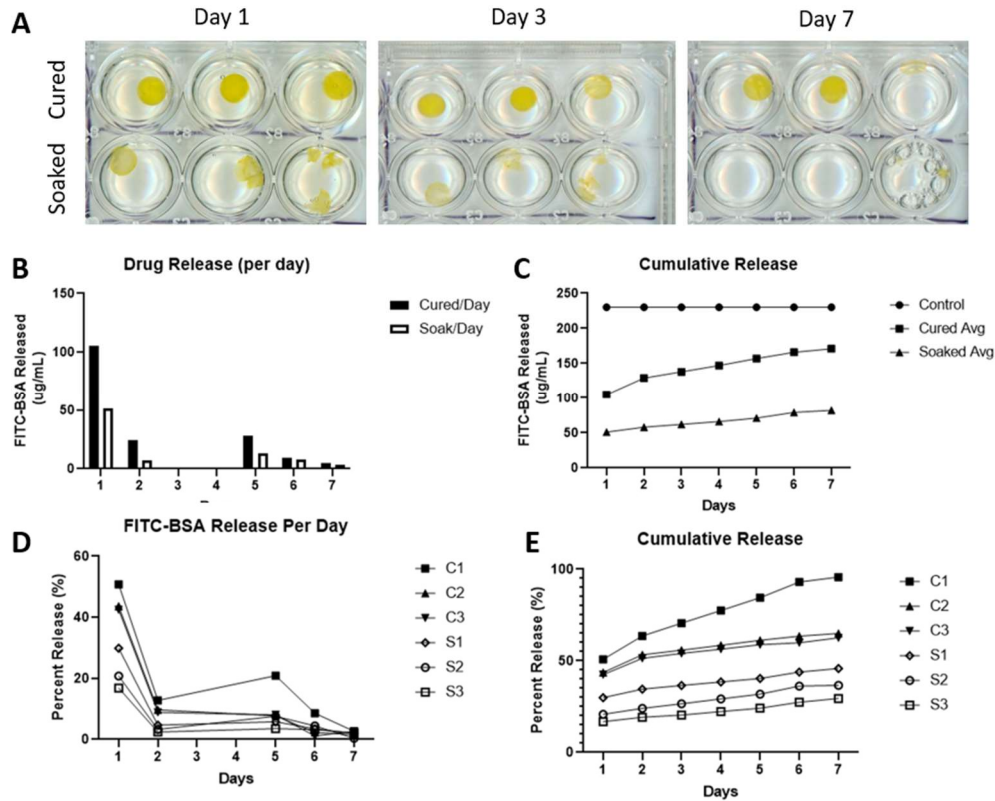


Figure 16. FITC-BSA Drug Release kinetics within Fibrin Gel. Gels submerged in 1xPBS for 1 week. **A:** EPSON Scanned images of each gel after time had passed (Cured – top, Soaked – bottom). **B-E:** Daily FITC-BSA release and cumulative release graphs. Data in percentage or µg/mL (n=3).

From the data it is shown that the cured scaffold has a higher average daily release compared to the soaked scaffold (**Figure 16B-E**). The soaked scaffold consistently had a lower release and this was visualized within the EPSON scans of the gels. By day 7, most of the soaked scaffolds were dissolved and thus the FITC-BSA along with it. The cured scaffolds were mostly intact besides sample 3 (**Figure 16A**). We can also assume that the soaked scaffold had a lower initial loading percentage since the scaffold is dissolved after 7 days with a lower cumulative release. The cured scaffold had a higher initial loading percentage and provided a more sustained release compared to the soaked scaffold. From

the graphs, the fibrin gel carrier exhibits an initial burst response followed by a sustained release in the PBS solution. We decided to administer FK506 through the cured method for the rabbit study. It is worth noting that in vivo, the fibrin gel will degrade rather quickly.

3.5.2 Animal Model Generation

One of the cornerstones of this project was the ability to translate results and findings clinically. We decided to do this within an animal model to evaluate whether our treatment could affect non-union rates. We decided to choose a rabbit model since the anatomy allows us to insert screws unlike a rodent model. To our knowledge, there is only 1 other study concerning ankle fusion in rabbits (insert source here). Due to cost constraints, we are evaluating the effectiveness of FK506 within 10 New Zealand white rabbits for a 4-week study. Our protocol for fusion involved the insertion of a 2.3mm diameter screw following the debridement of the articular cartilage and preparation of the joint space (**Figure 17A**). FK506 was then inserted within a carrier fibrin gel at 0.1mg/kg dosage (**Figure 17B**). Fusion was evaluated following screw fixation by the surgeon. Analysis will be completed following this study's completion. We will be primarily looking at non-union rate, histology, and bone growth within the fusion site. Protocol creation done by Dr. Jason Bariteau. Surgery done by Dr. Jay Patel and Hanna Solomon. Sample preparation done by Nicholas Huffman.

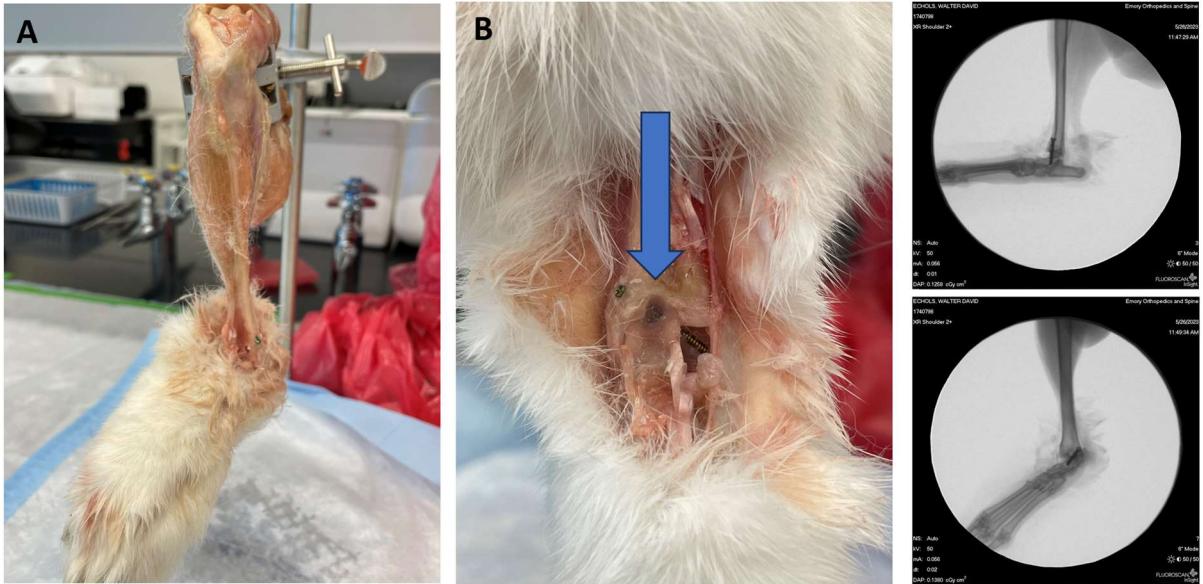


Figure 17. Sample photos of rabbit cadavers. A: Screw inserted into the medial malleolus (2.3mm diameter, 18mm length). **B:** 50uL fibrin gel injected into the joint space and allowed to form. Radiograph images provided to show screw depth and fixation.

CHAPTER 4. DISCUSSION

In this study, we demonstrated that FK506 induces significant osteogenesis within the Marrow Derived cell population and within the Human Mesenchymal Stem Cell line. We verified this observation through a multitude of assays including mineralization, ALP, rt-qPCR, nuclear localization, and μ CT quantification. These results were collected across multiple culture conditions and cell types which makes the analysis unique compared to traditional papers. Examination of osteogenesis under Basal Media and Osteogenic Media conditions can illustrate and present comparisons to clinical translational models. Overall, the results show that FK506 induces mineralization and expression of osteogenic genes within our 2D MDC culture model. Though it was discovered that there can be significant variation in outcomes related to different MDC donors. We identified rhPDGF-bb as a potential adjuvant to FK506-induced osteogenesis. Through the rt-qPCR results we determined that there may be a temporal aspect to the rhPDGF-bb treatment within early timepoints. In summary, we have identified that FK506 induces significant osteogenesis within our 2D culture models. Our 3D cultures suggest an alternative explanation that the combination of FK506 and rhPDGF-bb can cumulatively affect mineralization following 4 weeks of culture within hMSCs. The MDC culture did not reveal significant differences between culture groups.

As mentioned above, this is not a traditional paper on osteogenesis as it may seem. It is a journey to understand the full nature of what osteogenesis means to our fusion site. Since the far end goal of this research is to uncover the clinical capabilities of FK506 treatment,

it was our objective to make this translatable to arthrodesis treatment. We initially started off with evaluating osteogenesis within a Basal Media culture model. Basal Media (10% FBS) does not contain any osteogenic factors within the media. Primarily, we were interested in the raw osteogenic potential of FK506 within the MDCs. Our results indicate that FK506 does induce mineralization from Alizarin Red S staining at doses from 1.5 μ M to 6 μ M. From here, we decided to test for any potential adjuvants related to FK506 osteogenesis and identified rhPDGF-bb as an attractive candidate. The subsequent ALP assay found that though the combined treatment produced significant ALP, the individual treatments were not significant compared to the control. Since ALP is standardized to protein count, this poses the question of whether rhPDGF-bb treatment just proliferates many cells that produce a low osteogenic signal. This parallels directly with the proposed clinical explanation for rhPDGF-bb effectiveness within ankle arthrodesis treatment.

From here we decided to perform cultures within osteogenic media. This allows us to investigate FK506's effectiveness within a stimulatory environment similar to previous work done. Within the osteogenic medium we see that 1.5 μ M FK506 treatment resulted in the highest mineralization compared to the same assay within basal media. Additionally, the ALP assay showed that there is a significant difference between FK506 and control. One of the main observations is the intense red stain observed compared to the basal medium, showing that there is more mineral being produced.

The rt-qPCR results ended up giving us a lot more questions than answers for our specific applications. We were initially testing between different timepoints (3 and 14 days) in

order to understand the osteogenic differentiation timeline and whether different drugs affected early/late response. We began to see that rhPDGF-bb had a substantial effect on 72hr treatment compared to FK506, with higher expression levels than FK506 in a majority of genes. The combination of FK/PDGF did not seem to have this additive effect. For the 14-day expression, it was evident that FK506 had an increased effect compared to the rhPDGF-bb and FK/PDGF groups. Within the D35 MDC population, the FK506 group resulted in an increase within almost every gene. Most notably, the increased RUNX2 expression since that is the master regulator of osteogenesis. Overall, it is difficult to fully analyze the rt-qPCR results due to the donor differences and low enhancement of signal compared to the control. But it does provide an interesting snapshot into what can be considered a ‘responsive’ donor.

Another question to answer is the minimal differences seen between the control and the treatment groups within the alizarin stains. Though independent studies have verified FK506’s effect on osteogenesis, our results presented us with another question. One plausible explanation for this was that the osteogenic stimulus of the media was too high. Where any associated difference between the control and treatment would be negligible. This could have affected our hMSC staining results and ALP assay. The osteogenic stimulus used does contain FBS, which could be a source of variation between experiment runs. Additionally, the basal media contains 10% FBS. We performed an assay to test this for mineralization content, using our 1:4 ratio and 1:10 ratio and found that FK506 did result in a significant increase in mineral content. Although we can see

these differences within a cumulative assay such as the alizarin stain, the rt-qPCR results might not show a noticeable difference.

The micro-CT data is the best portrayal of osteogenesis that we can study. Since most mineralization happens after 2-3 weeks of cell culture, we wanted to actually measure this within a 3D model. We had no idea if mineral would even be detected by the micro-CT, and so we set a pilot experiment up for 4 weeks in order to see if we could detect changes. From the results we were able to see mineralization of the fibrin gel, which told us that we could obtain differences between treatment groups. Additionally, we changed the FK506 dosing from 1.5 μ M to 0.5 μ M because we had concerns over the high dosage for the longer culture period. Supplemental alizarin plates ran with 0.5 μ M compared 1.5 μ M yielded similar results. We then tested both MDCs and hMSCs to see if there were any differences between the treatment groups in their respective cell line. The MDC results did not show any differences between the treatment groups. This was surprising to us since we saw robust differences within the 2D culture plate groups. Within the hMSC group, the FK/PDGF treatment had the highest bone volume, tissue mineral, and BV/TV compared to the control. Looking at the graphs, it is easy to note that there are two data points that bring up the mean significantly. From this, the results of the study are not significant statistically. Though FK/PDGF treatment seems to provide the highest potential for bone growth out of the treatment options.

From the results, it is important to identify which metrics we have the most confidence in when determining our analysis. Cumulative assays such as ALP, Alizarin staining, and micro-CT show changes over the entire course of the culture. Whereas rt-qPCR results

and alizarin gel staining have inherent variability associated with the data. Though rt-qPCR provides an interesting snapshot of what the cell is doing at that exact timepoint, it can be hard to be consistent with the data output. The results are followed up by a western blot of the targeted pathway, but we did not have enough time to perform these assays. The alizarin staining of the gel is also variable. From the micro-CT reconstructed images, we can see that the mineralization is not homogenous within the gels. This can be due to cell migration and other factors that affect cell movement and proliferation. Sectioning a 20 μ M block out of a \sim 1000 μ M gel can result in variability since the entire area is not stained. This is where we have the most confidence in the micro-CT data for the calcification of the tissue.

4.1 Donor Variability

As said previously, one of the main goals of this study was to make it clinically translatable. Since we are concerned with ankle arthrodesis, we want to use cells that are in the fusion environment. This is the draw of using the MDC cell population, however there are some major limitations. MDCs have a variety of cell types within the population: mesenchymal stem cells, endothelial cells, and immune cells [52]. For our purposes, we are only concerned with cells that have an osteogenic potential. This limits our analysis to utilizing the MSCs and osteoblast/pre-osteoblastic cells within the assorted assays. Across our work we utilized three different MDC donors: D10, D35, and D38. These cells were isolated from bovine femoral condyles which can contain different ratios of each cell type listed above. This variability in cell composition can affect each run of our experiment and it is prevalent within our rt-qPCR results. This is highlighted

with the nuclear localization studies on the bovine MDCs in Figure 11. Since the localization is calculated on a per cell basis, any non-responding cell can affect the average greatly. In order to counteract this, we must be sure to use a high enough number of cells in order to ensure that non-responsive to responsive ratios are the same (assuming good mixing of the cells prior to seeding onto the well plate). From these efforts we can see a difference between the FK506 and CTRL groups. Future work will be to repeat the nuclear localization with hMSCs, along with Flow Cytometry data to fully investigate the MDC cell type.

The variability within the rt-qPCR results is also something to discuss. Across three different MDC donors and two timepoints we can really see responsiveness between donor groups. Primarily, we will focus on the consistency of the gene expression across the MDC groups. We start to see some trends within the 72-hour group, where FK506 results in consistent downregulation of OCN, OPN, and Col-1. However, RUNX2 and Osterix results yield a mix of results. For groups involving PDGF-bb, we see that RUNX2, ALP, and Osterix expression is drastically different in D35 than D10 and D38. Overall, the difference in expression levels between the three groups is something to highlight. Though the exact levels of upregulation/downregulation can be speculated, the donor dependent differences (think D38) are interesting to look at.

Concluding our work with the MDCs we wanted to shift to a less variable cell population such as the hMSCs in order to limit the variability that we saw from the MDCs. We decided to choose the hMSCs because they are a representative population of the fusion environment and can give us insight into the differentiating effects that FK506 or

rhPDGF-bb possess. Though the switch between MDCs and hMSCs didn't exactly give us the results we were expecting from our hypothesis, there are some interesting takeaways. Notably, the difference in alizarin staining intensity for the gels.

The donor variability is an interesting twist onto the results seen above as it can reinforce calls for personalized medicine. While the D10 and D35 donors exhibited mineralization and positive expression within rt-qPCR. The D38 donor did not. We could compare these donors to what surgeons' experience when working with new patients. A patient who has been smoking or is diabetic may fall into the D38 phenotype of bone healing. Whereas a young patient could be D10/D35. While debatably frustrating to work with from a science perspective, this can provide valuable insight into why non-union rates are so variable per patient. Future directions will include Flow Cytometry of the different donors to determine if there are significant differences in the cell populations of the donors. We hypothesize that difference donors have different percentages of MSCs/pre-osteoblasts and that can lead to functional differences seen within the assays done within this study.

4.2 Summary

In summary, we were able to identify FK506 as an osteogenic model within the MDC and hMSC cell lines. Our staining data and biochemical assays suggested that there should be more research into the cellular mechanisms revolving around rhPDGF-bb and osteogenesis in vivo. There also needs to be more in vivo work surrounding ankle fusion and current methods. Future work will involve additional assays concerning western blot

analysis and single cell sequencing to determine cell population differences between the MDC groups. It is hypothesized that the cell population differences will correspond to different healing phenotypes. Additionally, more research needs to be done concerning how the ‘fusion’ works within the joint space. Flow Cytometry of the MDC population would also be a valuable piece of information for our analysis. We currently don’t know mechanistically how joint fusion is different from long bone/fracture healing.

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