

Examining the Role of Ascorbic Acid in Prevention of Heterotopic Ossification

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Abstract

Heterotopic ossification (HO) is a condition in which mature bone forms in a non-osseous tissue following extreme trauma such as injury or surgery [1]. It is thought that a particular cell type known as mesenchymal stem cells (MSCs) are the main contributor to this condition. Here we identify muscle precursor cells (MPCs), a population of multipotent stem cells, as an alternative source of HO. The present study confirms this potential of MPCs to be induced into an osteogenic lineage, demonstrating significant increases in alkaline phosphatase (ALP) activity when MPCs were treated with 100ng/ml bone-morphogenetic protein-2 (BMP-2), ($p \leq 0.05$). These findings were used to develop the model for this study. The main objective of this study was to examine the efficacy of vitamin C (ascorbic acid) as a potential new prophylactic treatment for HO. Current prophylactic treatments include radiation therapy and the use of non-steroidal anti-inflammatory drugs (NSAIDs). Radiation therapy works based on the production of reactive oxygen species (ROS) which cause oxidative stress in the individual, thereby disrupting the mechanisms associated with HO. These preventative treatments however are either expensive and/or may result in non-union of normal bone or other harmful side-effects. As such, a new preventative treatment is needed for this condition. This study demonstrates that at doses of 200 μ M ascorbic acid functions as a pro-oxidant by producing a type of ROS; superoxide. When cells were treated with 200 μ M ascorbic acid in combination with 100ng/ml BMP-2, ALP activity did not increase. This suggests that ascorbic acid is able to block BMP-2-induced osteogenesis of MPCs. When cells were

treated further with an antioxidant, 1mM TEMPOL, in combination with 100ng/ml BMP-2 and 200 μ M ascorbic acid, alkaline phosphatase activity significantly increased. These data suggest that ascorbic acid can act as a pro-oxidant to create oxidative stress and prevent osteogenesis in MPCs. If administered at the appropriate dose, this treatment may function in a similar way to radiation therapy, by disrupting mechanisms involved with HO via oxidative stress, without the negative side-effects or harmful risks. The results of this study indicate that ascorbic acid should be further explored in animal models and clinical trials as a potential prophylactic treatment for HO.

Lay Summary

Faculty and students in the Department of Biology are bound together by a common interest in explaining the diversity of life, the fit between form and function, and the distribution and abundance of organisms. The purpose of this study was to investigate if vitamin C (ascorbic acid) could be used as a new preventative treatment for a condition known as heterotopic ossification (HO). Current preventative treatments include radiation therapy and the use of non-steroidal anti-inflammatory drugs (NSAIDs). Radiation therapy is effective because it disrupts the progression of HO through an increase in oxidative stress, however it has many harmful side effects and is expensive. The findings of this study show promising results as ascorbic acid proved to function in the same way as radiation therapy, by increasing oxidative stress, without the harmful and unwanted side effects. This suggests that ascorbic acid may be able to be used as a safer, cheaper, alternative treatment to what is currently available to prevent HO. In order to fully examine ascorbic acid as a new preventative treatment for HO animal studies and clinical trials would need to be conducted.

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Chapter 1. Introduction

1.1 Statement of Problem

Heterotopic ossification (HO) refers to the formation of mature bone in non-osseous tissue following extreme trauma such as injury or surgery [1]. It is also known as *myositis ossificans*. HO is triggered by a high-magnitude soft-tissue injury however the pathogenesis is largely unknown. Clinical signs and symptoms of HO typically arise as early as 3 weeks or as late as 12 weeks after the trauma has occurred [2]. There is increased prevalence of HO in military personnel due to combat-related or blast injuries. Studies by Potter *et al.* [3] suggest that HO occurs in 66% of lower-extremity amputees and 30% of upper-extremity amputees. The prevalence of HO in the civilian population is rare, however it is fairly common in very specific types of injuries including distal humeral fractures or ulnohumeral fracture dislocations. The incidence of HO ranges from 0%-49% following distal humeral fractures, and from 4% to 18% following ulnohumeral fracture dislocations [4]. The literature suggests that the cells responsible for causing HO, within muscle, are derived mesenchymal stem cells (MSCs) which have migrated to the region in response to tissue injury or trauma. MSCs are multipotent stromal cells which are located in many connective tissues of the body including adipose tissue, periosteum, synovial membrane, muscle, dermis, pericytes, blood, bone marrow, and trabecular bone. MSCs are able to regenerate cell types specific for these tissues such as adipocytes, myotubes, osteocytes, chondrocytes, fibroblasts and stromal cells. It

is thought that at time of injury a MSC of the myogenic lineage will adopt and follow an osteoblastic differentiation pathway in order to form mature bone in a non-osseous tissue, such as muscle. The pathogenesis of HO is largely unknown, however, multiple contributory factors have been proposed including prostaglandin activity, specifically PGE-2 (prostaglandin E2), along with hypercalcemia, tissue hypoxia, alterations in sympathetic nerve activity, prolonged immobilization, and imbalances between parathyroid hormone activity and calcitonin. These factors have all been shown to contribute to HO formation by enabling the improper differentiation of pluripotent MSCs into osteoblastic precursors [5],[6]. The focus of researchers studying HO has been almost entirely on these MSCs, even though they are not present within the muscle compartment at time of injury. An alternate stem cell which is being overlooked as a potential contributor to HO is known as a myogenic progenitor cell or satellite cell. Upon injury or trauma to skeletal muscle, satellite cells will proliferate and migrate to the injury site. Many of these cells will differentiate into myogenic precursors and contribute to muscle repair, while others will undergo asymmetric cell division to provide a sustainable pool of stem cells [7]. MPCs reside within the muscle compartment and because they are classified as stem cells, they have the potential to be induced into an osteoblastic lineage. This makes them a candidate for cells contributing to HO.

Current treatments are available to prevent HO including low-dose radiation therapy and non-steroidal anti-inflammatory drug (NSAID) therapy. Radiation therapy is thought to be effective because it induces oxidative stress in the

individual. This occurs by generation of reactive oxygen species (ROS) which then disrupt the mechanisms involved with HO [8]. Childs *et al.* found that following acetabular fractures, when radiation therapy was administered as a preventative treatment, only 5.3% of patients presented with HO. Conversely, when radiation therapy was not administered as a preventative treatment, 60% of patients presented with HO [9]. Similarly, NSAIDs have been demonstrated to reduce the risk of HO development by 57-59% if provided as a preventative treatment [10].

Although these treatments are for the most part effective, they are either expensive, difficult to administer, or provide unwanted side-effects. Radiation therapy has been shown to result in a 12-30% risk of nonunion of bone in addition to potential risk of carcinogenesis and azoospermia [11]. Additionally, radiation therapy has been developed for use in a civilian setting and is not realistic for individuals suffering from combat- or blast-related wounds in the military. NSAIDs may also impair healing of natural bone at the fracture site. A 29% incidence of nonunion of long bone fractures was observed after treatment with indomethacin (common NSAID) as a prophylactic treatment. Unfortunately, treatment of HO has only slightly changed in the last millennium. It is widely accepted that prophylaxis against HO is much more preferable than a later treatment of symptomatic lesions. This has led scientific research in recent years to focus toward prophylaxis and not treatments [3]. The use of radiation therapy as a preventative treatment for HO is most common however due to factors

outlined above there is a need for the development of a more suitable preventative treatment for individuals who could potentially suffer from HO.

1.2 Significance of Study

The term satellite cell describes a specific type of stem cell which is required for the growth, repair, and maintenance of adult skeletal muscle. These cells have been given their name based on their anatomical location on the surface of the myofiber beneath the basal lamina [12]. Once activated by injury or isolated from the muscle, these cells can rapidly expand, the progeny of which are termed muscle precursor cells (MPCs). These MPCs are multipotent meaning they are capable of differentiating into a variety of cell types including myoblasts and osteoblasts. Because of the multi-potency of these MPCs and their anatomical location within the muscle compartment, they may be a potential candidate for cells contributing to HO. As previously mentioned, these cells are overlooked in the literature as a source for HO. However, it is entirely possible that muscle cells are capable of differentiating into bone cells based on what is known about C2C12 cells. C2C12 cells are murine myoblasts derived from satellite cells and are commonly used to study myogenic processes. Additionally, these cells are classified as a pre-osteoblastic cell line which can be induced to undergo osteogenesis following the appropriate stimulation [13]. This suggests that muscle derived cell types can form osseous tissue.

Ascorbic acid, also known as vitamin C or ascorbate, is an essential dietary nutrient which is required for various biological functions including the biosynthesis of collagen [14]. At physiological or normal concentrations, ascorbic

acid functions as a water-soluble antioxidant, scavenging harmful radicals and protecting cells against oxidative damage caused by ROS [15]. Conversely, pharmacologic or increased doses of ascorbic acid have been shown to induce toxicity and oxidative stress in cells by production of ROS, indicating it also functions as a pro-oxidant [16], [17], [18], [19]. Therapies which have been designed to increase oxidative stress, using methods such as radiation therapy in combination with pharmacologic ascorbic acid, are being investigated as a method to preferentially sensitize tumour cells versus normal cells via metabolic oxidative stress [17],[18]. Ascorbic acid may exhibit its pro-oxidant actions and help the body's free radicals to destroy tumours in their early stages [19]. Based on these properties, and at the appropriate dose, ascorbic acid could potentially function as a preventative therapy for HO in the same mechanism as radiation therapy through production of ROS, without the harmful risks or side-effects. In 1992, a 12-year old patient suffered from myositis ossificans progressiva (MOP), which is an autosomal dominant condition involving severe heterotopic bone formation. The individual began treatment with large doses of ascorbic acid and eventually their symptoms improved [20]. Since then, ascorbic acid has either been only minimally, or not at all, reviewed in the literature as a potential treatment (prophylactic or other) for HO. Currently, most data suggest that very large doses of vitamin C are not toxic and will not cause serious adverse health effects in humans [21], [22]. Additionally, it is inexpensive as well as easy to administer, making it an ideal candidate for military personnel as well as use in a civilian setting.

1.3 General Research Question

The overall aim of this study is to establish *in vitro*, the efficacy of ascorbic acid as a preventative treatment for heterotopic ossification.

1.4 Specific Aims

1. Investigate the potential of muscle precursor cells (MPCs) to be induced into bone lineage.

MSCs are well studied in literature as a candidate for HO. These cells are believed to migrate to the tissue region following trauma and adopt an osteoblastic lineage leading to HO. A common model used to study HO with MSCs involves osteogenic induction with bone morphogenic proteins (BMPs), specifically BMP-2. What is overlooked in literature are MPCs which are already present within the muscle compartment at time of injury. MPCs have the ability to undergo asymmetrical cell division. Therefore, they have the ability to self-renew, classifying them as a stem cell. As such, these cells are capable of differentiating into multiple different cell lineages including osteoblasts [7]. The first aim is therefore to determine if MPCs can be induced into an osteogenic lineage (bone lineage) in the same way that C2C12 cells, which are both a myogenic and pre-osteoblast cell line, are in the literature using BMP-2 [23] .

Hypothesis:

MPCs are sensitive to induction of an osteoblastic lineage with BMP-2 similarly to the C2C12 cell line.

2. Determine the effects of ascorbic acid (vitamin C) on osteogenesis of MPCs

Ascorbic acid is often considered an antioxidant, however under the appropriate concentrations and in the presence of transition metals such as copper and iron, ascorbic acid functions as a pro-oxidant. This pro-oxidant potential of ascorbic acid results in oxidative stress by generation of ROS. These ROS may then have the ability to disrupt mechanisms involved with HO [24], [25]. The second aim is to therefore to determine how high doses of ascorbic acid will affect the osteogenic ability of MPCs.

Hypothesis:

At the appropriate concentration, ascorbic acid will prevent MPCs from undergoing BMP-2 induced osteogenesis.

3. Investigate if the effects of ascorbic acid on MPCs, which have been induced to undergo osteogenesis by BMP-2, are due to pro-oxidant activity

If the actions of ascorbic acid in reducing osteogenesis are due to pro-oxidant activity, then they should be prevented by addition of antioxidants such as 4-Hydroxy-TEMPO (TEMPOL) or polyethylene glycol-catalase (PEG-Cat). As viewed in Figure 1, catalase will convert H_2O_2 produced by ascorbic acid to H_2O and O_2 within the blood and extracellular fluid. It therefore can be utilized to indicate if a reduction in H_2O_2 will reverse any potential effects observed by ascorbic acid. TEMPOL is a redox-cycling (catalytic), metal-independent, and membrane-permeable antioxidant. It will promote metabolism of O_2^- similarly to superoxide dismutase (SOD), therefore it is considered a SOD mimic [26].

TEMPOL can therefore be used to indicate if superoxide scavenging prevents the effects of ascorbic acid. Investigating the antioxidant roles of PEG-Cat and TEMPOL may provide insight as to the type of ROS, if any, being produced by ascorbic acid at high doses in BMP-2 treated MPCs. The third aim is therefore to investigate through which mechanism ascorbic acid blocks osteogenesis of MPCs.

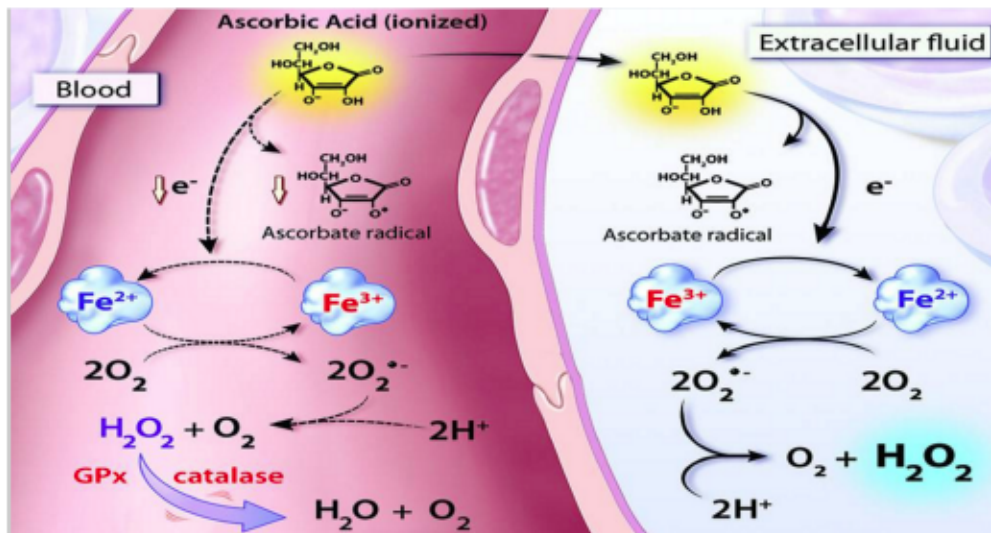


Figure 1. **Proposed mechanism by Chen, et al. in which ascorbic acid acts as a pro-oxidant** [27]. In the extracellular fluid, ascorbic acid will lose one electron and produce ascorbate radical ($Asc\bullet$). The electron will reduce a transition metal such as iron (Fe^{3+} to Fe^{2+}). The Fe^{2+} then donates an electron to oxygen (O_2), forming superoxide ($O_2^{\bullet-}$) and O_2 . This is followed by the generation of hydrogen peroxide (H_2O_2) which will diffuse across the cell membrane and cause oxidative damage to cellular proteins, lipids and DNA. In the blood, $Asc\bullet$ will be inhibited by red blood cell membrane-reducing proteins and/or by large plasma proteins not found in the extracellular space. Any H_2O_2 produced will be immediately destroyed by plasma catalase [27].

Hypotheses:

At high doses (50-500 μ M) ascorbic acid will function as a pro-oxidant.

Addition of the antioxidants TEMPOL and PEG-Cat will reverse these pro-oxidant effects, thus reversing any osteogenic inhibition observed by the ascorbic acid treatment.

4. Investigate the effects of ascorbic acid on myogenic capacity and proliferation of MPCs.

The role of ascorbic acid specifically on myotube formation and proliferation of MPCs *in vitro* has not been investigated in the literature. Ideally high doses of ascorbic acid will prevent MPCs from undergoing osteogenesis. However, it is important that ascorbic acid does not impair or negatively influence normal myogenic differentiation of MPCs as well. The fourth aim is therefore to determine the effects of ascorbic acid on the myogenic ability and proliferation of MPCs.

Hypotheses:

Ascorbic acid will not interfere with myogenic mechanisms associated with *in vitro* MPC differentiation and production of mature myotubes and will not negatively affect proliferation of MPCs.

5. Determine if there is a difference in osteogenic capacity between MPCs and a subpopulation of cells where the highly myogenic cells have been removed.

The MPC cell pool is heterogeneous because of the ability of MPCs to undergo asymmetric cell division. Some cells will self-renew to maintain the satellite cell pool, while others will continue to proliferate, eventually adopting a myogenic fate [7]. In culture, when MPCs begin to differentiate, most cells elongate and begin to fuse to form myotubes, however as outlined below in Figure 2, some cells do not. This suggests that perhaps there is a subpopulation of cells present within the MPC cell pool which have a lower myogenic capacity compared to those forming myotubes.

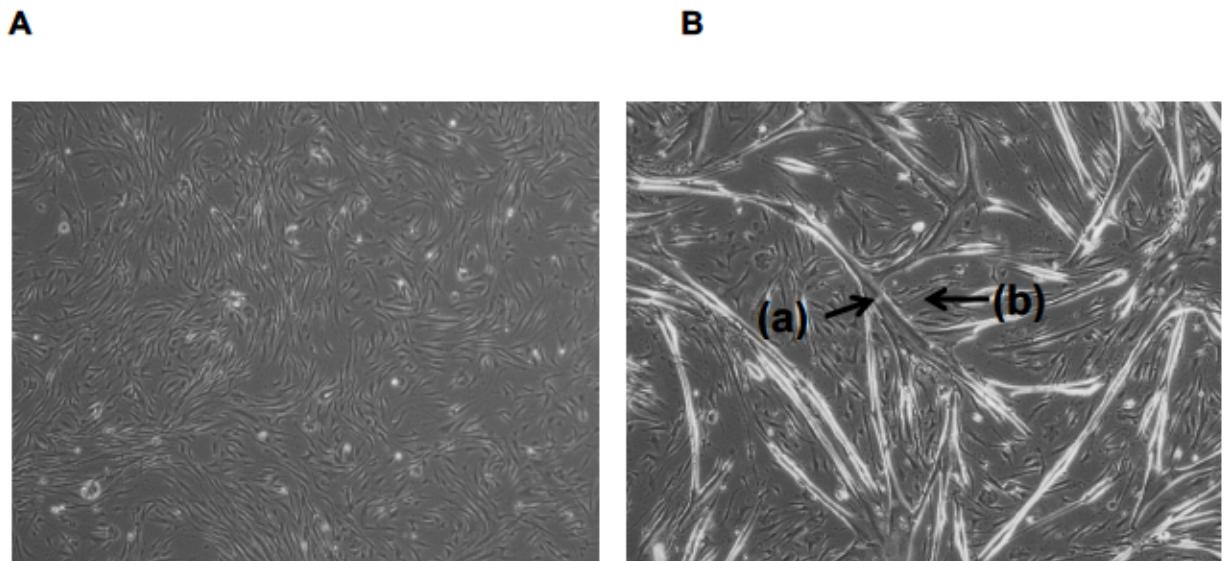


Figure 2. **Differentiation of MPCs.** Panel A demonstrates a confluent monolayer of MPCs prior induction of differentiation. Panel B shows formation of elongated myotubes capable of contraction (a). A subpopulation of cells exists below which have not elongated and produced myotubes (b).

Hypothesis:

There will be a difference in osteogenic capacity between MPCs and a subpopulation of cells where the highly myogenic cells have been removed. This subpopulation of cells may be more likely to undergo osteogenesis and less likely to undergo myogenic differentiation.

Chapter 2. Literature Review

2.1 Heterotopic ossification overview

The term *heterotopic ossification* refers to the abnormal formation of mature, lamellar bone in a non-osseous tissue. This can lead to loss of motion and severe pain in the limb or region. If translated to the Greek (*heteros* and *topos*) and Latin (*ossification*) origins, heterotopic literally is defined as “bone formation in another location” [3]. The formation of HO has been observed following extreme trauma and/or surgery such as: total hip arthroplasty, acetabular and elbow fracture surgery, electrocution and burn injuries, and traumatic brain injury or spinal cord injury [28]. There are a few risk factors associated with HO including head injury, duration of intubation, Injury Severity Score (ISS), delay from injury to surgery or fixation, burn, spinal cord injury, and number of operations. [4].

2.1.1 Incidence of heterotopic ossification

Based on political discrepancies between Iraq and Afghanistan, studies have recently reported that the incidence of HO in the residual limbs of combat-related amputees is as high as 63% in patients [25]. This is much higher in comparison to reports of civilian trauma. Until recent years, the occurrence of HO in the residual limbs of amputees was rarely described in the literature. Possible reasons for increased prevalence of HO after extremity blast amputations are likely due to the increased use of improvised explosive devices. These devices cause a high-energy mechanism of injury. In addition, military trauma orthopedic surgeons use aggressive limb-salvage techniques to preserve and maintain

function of as much of the limb as possible. Therefore, more military personnel are surviving high-blast injuries, however they have severe extremity trauma which would have been fatal to those in the past [29],[30]. This type of severe injury in the limbs of amputees is clinically significant because it can cause severe limb pain and skin breakdown, leading to increased need for pain medication. Additionally, HO can cause issues with prosthetic fitting and use. Potter and colleagues [25] note that the prevalence of heterotopic bone formation is significantly greater in human blast amputees than in human non-blast amputees. This suggests that there are specific factors regarding an explosive mechanism of injury which lead to HO that do not normally occur with non-blast mechanisms.

Although not as common in a civilian population, HO can occur in both contact and noncontact sports. Myositis ossificans traumatica (MOT) for example is a condition observed in adolescents and young athletes. It results in hematoma formation resulting in nonneoplastic bone or cartilage development following blunt muscle trauma [31]. In a particular civilian case, a 20-year old hockey player received an acute blow to the lateral thigh. This resulted in significant swelling, preventing the individual from skating. At the time of injury, the individual was treated with a deep tissue massage, active stretching of quadriceps, heat, and a tight elastic bandage wrapping. He was then allowed to continue in the hockey game. An X-ray was taken four hours after the injury and revealed a large amorphous radiopaque mass in the thigh. This study suggested that the formation of this mass was due to inappropriate treatment with deep

tissue massage and heat, at the time of injury, and led to a natural progression of heavy ossification roughly 3 months later [31]. It should be noted that typically MOT takes 3-4 weeks after initial injury to be visible radiographically [32].

Matsumoto *et al.* [33] reported an incidence of HO in civilian amputations to be 22.8%. After most traumatic injuries in the civilian population, HO formation is relatively rare in the absence of head injury. Even after traumatic brain or spinal cord injury, HO develops in only 20% and 11% of patients [34]. HO formation exceeds 50% following a femoral shaft fracture with associated head injury [35].

Incidence of HO increases by 57% in patients which sustain a polytraumatic blast injury [25]. The most common sites for the formation of HO are after an open-reduction internal-fixation (ORIF) for acetabular fracture and the hip after total hip arthroplasty (THA). The incidence of HO following THA has been reported to be between 5 and 90%. Only 3-7% of these patients develop clinically significant HO however. Incidence of HO is between 3.8 and 39% following primary total knee arthroplasty (TKA) [6]. In summary, proper care and prophylactic treatments are necessary when there is a risk of HO development in contact sports as well as surgery or combat-related injuries.

2.1.2 Current prophylactic treatment of heterotopic ossification

Studies suggest that one of the most important factors for preventing HO involves actions taken immediately after injury. Studies by Douglas and colleagues [4] suggest that HO occurs more frequently after severe injury or trauma to the elbow. Treating individuals with distal humerus fractures and elbow dislocations improperly has the potential to cause HO. These researchers

report that increased delay from injury to fixation is an independent risk factor for clinically significant HO and they encourage the consideration of HO prophylaxis for patients, particularly in elbow fracture dislocations [4]. Ilahi *et al.* [36] report that a delay of 24-48 hours from injury to definitive fixation led to an increase in HO following elbow trauma. Of the 41 elbow injuries discussed, none of the fractures fixed within the first 48 hours developed grade 2 through 4 HO, compared with 33% of patients treated after 48 hours.

Unfortunately, treatment of HO has only slightly changed in the last millennium. It is widely accepted that prophylaxis against HO is much more preferable than a later treatment of symptomatic lesions. This has led scientific research in recent years to focus toward prophylaxis and not treatments [3]. Current prophylaxis for HO includes local radiation therapy and NSAIDs. The use of radiation therapy as a preventative treatment for HO is most common. Typically, a dose of 700-800 centigray divided within 4 days of trauma or operation is administered to the patient [8]. To put this in perspective, a typical radiation therapy treatment for cancer consists of daily fractions (doses) of 1.5Gy to 3Gy (150-300 centigray) given over several weeks [37]. Stein *et al.* [38] administered a postoperative single dose of 700-centigray radiation therapy within 72 hours of surgery to individuals who sustained a high-energy trauma to the extremity causing elbow damage. They discovered that 27% of patients had radiographic evidence of HO formation however 91% were without functional limitations. These data suggest that a single dose of 700-centigray radiation therapy within 72 hours postoperative may lessen the functional loss from HO

formation without affecting healing of the fracture site. Caution must be executed when using radiation therapy as a preventative treatment for HO however there is a possibility of the individual developing radiation-induced sarcomas. These sarcomas are malignant tumours composed of connective and other non-epithelial tissues. Such dangers must be considered when administering radiation doses to patients on a benign condition such as elbow trauma [38]. Both radiation therapy and NSAIDS have been shown to effectively prevent primary HO, however studies suggest that there are fewer serious side effects observed with radiation therapy [39]. It was observed in the early 1500's [40] and later in the 1970's [41] that radiation inhibited bone repair in rats. These effects were even more evident when the radiation was administered closer to the time of the fracture. As such it was hypothesized that early osteoprogenitor cells involved in bone repair were more radiosensitive than more mature cells observed later in bone development [11]. Today radiation therapy is used prophylactically both pre- and postoperatively for prevention of HO following trauma or operative treatment. Childs *et al.* report that out of 263 patients who experienced a traumatic acetabular fracture, HO was observed in 5.3% of patients who received radiation therapy while 60% of those who did not receive treatment developed some degree of heterotopic bone [9]. Radiation therapy is generally administered within 24 hours preoperatively to 48 hours postoperatively in patients at risk for HO. Studies suggest that there is no statistically significant difference between preoperative (<4 hours preoperatively) and postoperative (<72 hours postoperatively) radiation therapy [42],[43]. There are several

potential side effects of radiation therapy including the risk of carcinogenesis. Radiation therapy is administered in low doses and there are currently no reports of radiation-induced tumour formation after radiation therapy for HO prophylaxis. Reasons for this may be however due to an older patient population. The latency period for induction of malignancy following radiation therapy is roughly 15-24 years, therefore it is possible that not enough patients survive long enough after treatment for carcinogenesis to take place [11]. An additional risk involved with radiation therapy is nonunion of bone. Bone nonunion rates range from 12-30% after radiation therapy in literature [11]. A final concern with radiation therapy as a prophylactic treatment directly involves the testis. Low doses between 20 and 70cGy of radiation have been shown to cause oligospermia (low sperm concentration) in animals, while doses of 120cGy have been shown to cause permanent azoospermia (absence of sperm in semen) [11].

The other accepted form of HO prophylaxis is the use of NSAIDs. NSAIDs prevent HO formation by inhibiting the production of prostaglandins, specifically prostaglandin-E₂. Prostaglandin-E₂ is a hormone-like substance which has been reported in the literature to increase bone formation [6]. The use of NSAIDs pre-operatively was shown to reduce the risk of developing HO bone formation by 59% over the placebo group [10]. A similar group reports a 57% reduction in the risk of HO when NSAIDs were used as a prophylactic treatment [44]. This particular group suggests, based on their statistical data, that for every 100,000 total hip arthroplasties performed in the US each year, perioperative NSAID treatment can prevent anywhere from 10,000 to 20,000 cases of HO bone

formation. Indomethacin specifically has been shown to help prevent HO, decrease the extent of development of HO, and contribute to preventing inflammation associated with unwanted HO [45], [46], [47],[48]. A major problem with NSAIDS, such as indomethacin, is risk of increased perioperative bleeding [49]. A study by Fransen *et al.* reports an overall risk of HO reduction by 31% with the use of ibuprofen, however there was a significant increase in major bleeding complications with this treatment [50]. Another major problem associated with the use of NSAIDS for HO prophylaxis is that, while they prevent new heterotopic bone from forming, they also may impair the healing of normal bone at the fracture site. In a study by Burd and colleagues [47] a 29% incidence of nonunion of long bone fractures following indomethacin (NSAID) prophylaxis was observed, while the arm that received radiation was only 7%.

In addition to the use of NSAIDs and radiation therapy, early and consistent active range of motion exercises have been demonstrated to help prevent HO formation in the elbow [51]. Other options for treatment have been considered for HO as well, however results are inconclusive. Vitamin A or retinoic acid has been demonstrated to inhibit chondrogenesis [52], which is a prerequisite for bone formation. As such, isotoretinoin (13-*cis*-retinoic acid) has been explored as a preventative treatment for HO. Clinical trials revealed mixed and unclear results however and resulted in unwanted side effects including skin problems and hair loss which discouraged long-term treatment [53]. In summary, a new preventative treatment which will not impair normal bone repair at the

fracture or injury site, or provide other harmful side-effects, but will prevent formation of heterotopic bone, is needed.

2.1.3 Pathogenesis of heterotopic ossification

Chalmers *et al.* state that that in order for bone induction to occur in soft tissues, three conditions must be present: an inducing agent, an osteogenic precursor cell, and an environment which is suitable for osteogenesis [54]. The pathogenesis of HO is unknown however, studies suggest a distinct series of events take place upon formation of HO in soft tissues. First, progenitor cells in the muscle compartment will proliferate and generate a fibroproliferative lesion. This lesion is rich with collagen and other matrix proteins. Many of these proliferating cells have the ability to differentiate into osteo-progenitors which can then be stimulated to undergo osteogenic differentiation. Because muscle tissue is conducive to bone under the appropriate signaling environments, these progenitor cells can undergo endochondral or membranous ossification. Eventually this will lead to heterotopic bone formation within the muscle [28].

Although the pathophysiology of HO is largely unknown there are few local changes which occur at the injury site during HO formation. Because NSAID's are useful in preventing HO, it has been suggested that prostaglandins may play an important role in HO. Prostaglandins are local mediators of inflammation and bone remodeling. There have been time-dependent changes observed during endochondral ossification in the profile of specific tissue prostaglandins including PGE2, PGF2alpha, thromboxane B2, and 6-keto-PGF1alpha [55]. In a rabbit model of HO, Bartlett and colleagues observed increase in PGE2 and

PGF2alpha within 24 to 48 hours of experimental HO induction [56]. Based on these findings they suggest inflammatory processes may be important in HO formation and focus on the role of prostaglandins in that process. Additionally, they suggest that, because of the potential role of prostaglandins, in order for effective prophylaxis of HO with NSAIDs, treatment must begin almost immediately following the initial tissue trauma or injury.

In addition to changes in prostaglandins, there have been other local changes observed with HO. Studies have suggested that both physiological and pathological mineralization is initiated by matrix vesicles. The mineral phase formed in skeletal tissue is basic calcium phosphate (BCP) crystals. Matrix vesicles, membrane-enclosed particles, will initiate the mineralization process in growth plate cartilage and bone. They will then be released from the plasma membrane of mineralization-competent cells into the extracellular matrix. Within these vesicles is a low concentration of free calcium and inorganic phosphate ions therefore there is a constant influx of mineral ions into the vesicles. This leads to rapid mineralization. Once the mineral has reached the appropriate size it will cause the vesicle membrane to burst and continue to grow in the extracellular matrix. [57].

Extracellular changes in the concentration of inorganic phosphate and pyrophosphate/inorganic phosphate homeostasis have also been suggested to play an important role in the regulation of ectopic bone formation. Inorganic phosphate may be an important component of BCP crystals or it may function as a signaling molecule to regulate differentiation and mineralization events [58].

Extracellular inorganic phosphate and membrane transport proteins Pit-1 and Pit-2 have been shown to initiate the transdifferentiation of vascular smooth muscle cells into an osteoblast-like phenotype. This eventually led to matrix mineralization [59]. Other studies have demonstrated that high concentrations of extracellular inorganic phosphate will stimulate the apoptosis of osteoblasts and chondrocytes [60].

A final factor which may play a role in HO involves programmed cell death or apoptosis. When apoptosis occurs there is also release of apoptotic bodies. Phosphatidylserine is located on the outer membrane surface of apoptotic bodies due to exteriorization. However, phosphatidylserine is located in the inner region of the matrix vesicle membrane. It has been demonstrated to bind calcium and has been associated with the mineral phase in matrix vesicles [61], [62]. These results suggest that apoptotic bodies may play a role in physiological and ectopic mineralization. This may happen by the accumulation of calcium and inorganic phosphate on the outer surface of apoptotic bodies through their external phosphatidylserine. In conclusion, pathogenesis of HO or ectopic bone formation in general is poorly understood. However, understanding the roles of various factors in regulating physiological mineralization may provide insight as to strategies to prevent HO.

2.1.4 Mesenchymal stem cells: a suggested cell source for heterotopic ossification

MSCs are multipotent stromal cells believed to be involved in HO formation. MSCs promote wound-healing and regeneration of surrounding tissues by:

migrating to the site of injury, promoting repair and regeneration of specific damaged tissues described previously, modulating immune and inflammatory responses, stimulating the proliferation and differentiation of resident progenitor cells, and secreting trophic factors which are important in wound-healing and tissue remodeling [6],[7]. It has been suggested in the literature that following an appropriate stimulus such as injury, trauma, or surgery, MSCs will migrate to the damaged tissue. Because they have been shown to be capable of myogenesis [65], [66], [67], some of these cells may be contributing to normal muscle repair. Others however, may differentiate into osteoprogenitor cells. These osteoprogenitor cells can then be stimulated to undergo osteogenic differentiation. Under the appropriate signaling environments, these cells will then have the potential to undergo ossification and form mature bone in non-osseous tissue, leading to HO [38],[68]. An illustration of HO formation can be viewed below in Figure 3.

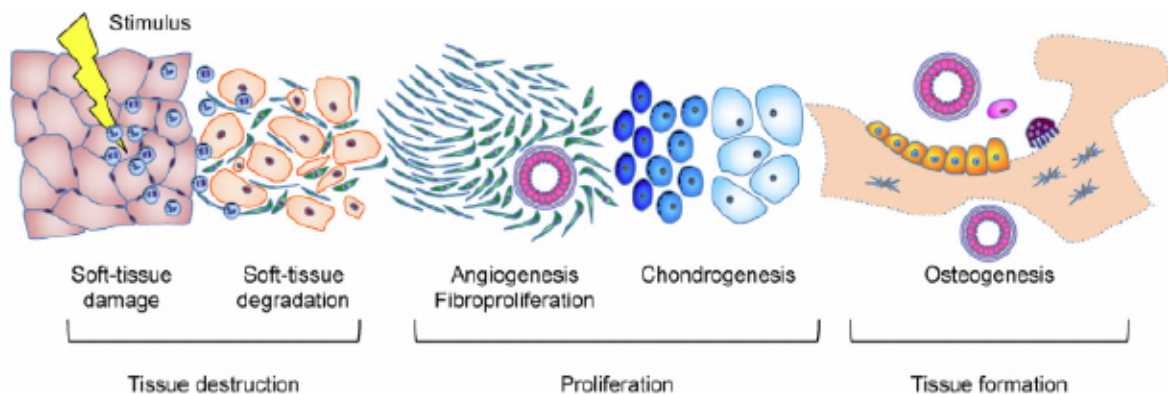


Figure 3. **Proposed illustration of the multi-stage process of HO formation** [69]. A schematic illustration of the multi-stage process of HO formation is illustrated. HO formation is believed to begin with inflammation resulting from a particular stimulus including trauma or surgery. The presence of inflammatory cells such as lymphocytes, macrophages and mast cells within the skeletal muscle and connective tissue are associated with damage to skeletal muscle

cells and tissue hypoxia. Both of these factors are capable of triggering proliferation of fibroblastic cells (undifferentiated mesenchymal cells). Tissue degeneration results in recruitment and activation of fibroblastic cells which differentiate into chondrocytes. This cartilage is eventually replaced by mature bone, resulting in HO within the tissue [69].

As previously stated, MSCs are multipotent. This means they are capable of differentiating into a variety of different cell types within the body. These cell types are outlined below in Figure 4. This characteristic is one of the reasons they are considered a potential source of HO.

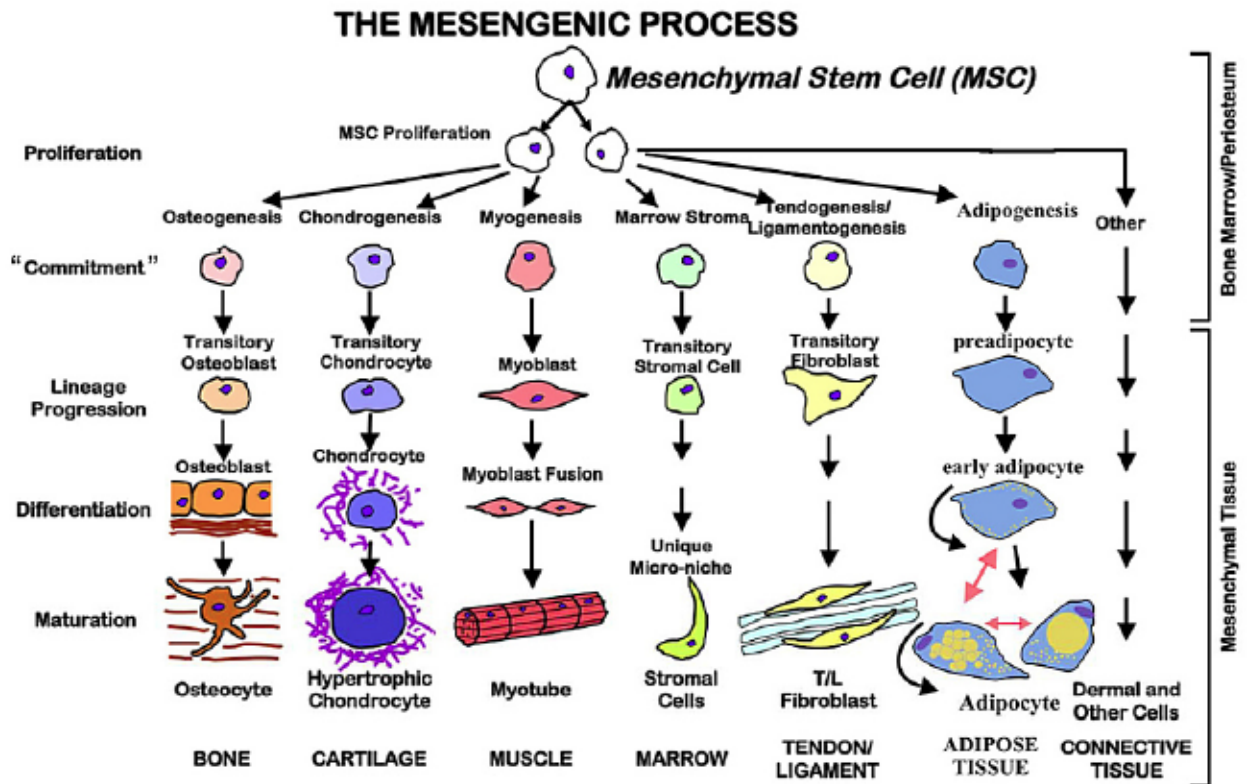


Figure 4. **Mesenchymal stem cell (MSC) differentiation.** [70] MSCs are capable of differentiation into a variety of different cell types. Such cell types include osteoblasts, myocytes, chondrocytes, and adipocytes. These cell types are capable of generating various tissues within the body including bone, muscle, cartilage, bone marrow, connective tissue, tendon/ligaments, and fat [70].

MSCs are located in a variety of areas of the body including whole bone marrow, however the concentration of them in whole bone marrow is fairly small. Estimates by Gronthos and Simmons [71] suggest 1 cell per $1-20 \times 10^5$ mononuclear cells while Pittenger and colleagues [72] suggests $1-10 \times 10^5$ nucleated cells in whole bone marrow. Proliferation and differentiation of MSCs is controlled and regulated by the Mitogen-Activated Protein (MAP) kinase pathway [73]. BMPs influence MSCs additionally through use of heteromeric type I and type II receptor complexes. These cause activation of intracellular proteins as well as MAP kinase. Upon BMP binding to the type I/type II receptor, an intracellular signal is produced and transmitted to the nucleus to adjust gene expression of certain proteins. This eventually leads to osteogenic differentiation of the cell [74], [75]. MSCs have been demonstrated by Ferrari and colleagues [66] to migrate from bone marrow to skeletal muscle to aid in repairing muscle when needed, such as during an injury. Following a fracture or bone injury which results in an inflammatory phase, there is a mesenchymal and angiogenic activation phase. Blood vessels as well as MSCs are recruited to the area where they begin to proliferate. MSCs will then begin to differentiate into chondrocytes or osteoblasts. Osteoblasts will form bone through a mechanism known as intramembranous ossification, while chondrocytes will proliferate, hypertrophy, and mineralize. New bone is then deposited onto the cartilaginous matrix via endochondral ossification. As such, MSCs are a critical factor in the process of bone healing and their actions can be manipulated to improve healing [76]. It is based on these characteristics of MSCs that makes them a popular candidate for

a source of HO. What is currently being overlooked are other potential sources of HO such as MPCs. MPCs reside within the muscle compartment and because they are classified as stem cells, they have the same potential to be induced into osteoblastic lineage as MSCs migrating to the region.

2.1.5 Satellite cells: an alternative cell source for heterotopic ossification

As previously described, MSCs are the believed cell source for HO, however there is another cell candidate which is completely overlooked in the literature. This type of cell is known as a satellite cell. Adult skeletal muscle in mammals is easily able to repair after injury under normal circumstances. This form of striated muscle tissue accounts for ~40% of adult human body weight. It is composed of multinucleated contractile muscle cells known as myofibers. During development, myofibers are formed by fusion of mesoderm progenitors known as myoblasts. Myofibers will grow upon fusion of satellite cells. Satellite cells are skeletal muscle stem cells which are crucial to the regenerative potential of skeletal muscle [77]. A stem cell is defined as an undifferentiated cell within the body with the ability differentiating into a wide range of specialized cells as well as undergoing self-renewal. The term 'potency' can be used to describe the capability of such a stem cell to differentiate into specialized cell types. There are five classes of potency: totipotent, pluripotent, multipotent, oligopotent and unipotent. Totipotent stem cells have the ability to differentiate into embryonic and extraembryonic cell types. Pluripotent stem cells can differentiate into almost all cells of the body including those in any of the three germ layers. Multipotent stem cells can differentiate into a number of cells from a single germ layer, while

oligopotent stem cells can differentiate into only a few select cells. Finally, unipotent cells can only differentiate into one cell type, their own, however they are capable of self-renewal [201].

A muscle satellite cell is defined based on its anatomical location relative to a myofiber. This is illustrated below in Figure 5. Once satellite cells are isolated from the muscle and cultured, their progeny are known as MPCs, which are the focus of this study.

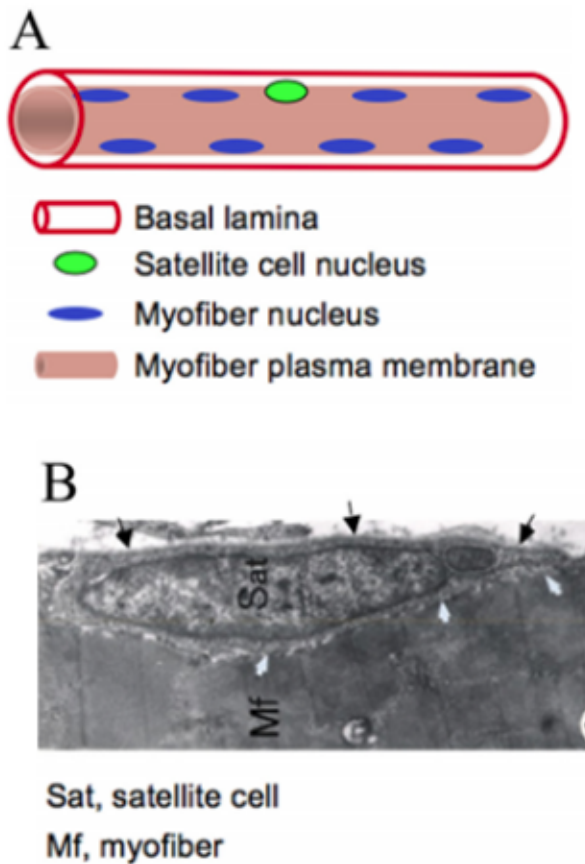


Figure 5. **Anatomical location of adult satellite cells** [12]. Satellite cells are located beneath the basal lamina of myofibers. These cells are required for the growth, repair, and maintenance of adult skeletal muscle [12].

Satellite cells supply myonuclei to growing myofibers until they enter a quiescent state in mature muscle. These cells become activated from this quiescent state when they are needed for muscle maintenance, hypertrophy, or muscle repair. The quantity of satellite cells differs between muscles, myofibers types, developmental stages, and species. Generally, satellite cells account for 2-7% of the sublaminal nuclei on myofibers in adult muscles [78]. Additionally, satellite cells are more localized in the ends of the myofibers, where longitudinal growth of skeletal muscle occurs [79]. In order to allow for muscle regeneration upon injury or trauma, the body has systems in place to maintain a viable cell pool. Therefore, satellite cells must be capable of self-renewal. Satellite cell self-renewal may be asymmetric, meaning one daughter cell is committed to differentiation while a second daughter cell continues to proliferate or becomes quiescent [7]. Studies have demonstrated that the Wnt signaling pathway plays a large role in the regulation of satellite cell self-renewal [80]. As previously described, satellite cells exhibit heterogeneity in respect to their cell fate potential. These cells are capable of differentiating into multiple mesenchymal lineages including myocytes, adipocytes, and osteocytes [81]. This characteristic suggests that satellite cells functionally resemble bone marrow-derived MSCs. Therefore, they have the same potential to be a source for HO as MSCs migrating to the injury site.

In order to contribute to muscle maintenance, regeneration and hypertrophy, satellite cells must first be activated from their quiescent (G_0 phase) state. This activation occurs following some type of muscle injury. Proliferating

satellite cells and their progeny are known as MPCs or adult myoblasts. Small injuries may lead to slight proliferation of activated cells, while major trauma can recruit greater numbers of satellite cells and promote a prolonged episode of proliferation prior to differentiation. These MPCs are activated by proximal signals from the muscle niche, microvasculature, and inflammation. Once activated, these cells contribute to regeneration of new myofibers following cell division and proliferation as depicted in Figure 6.

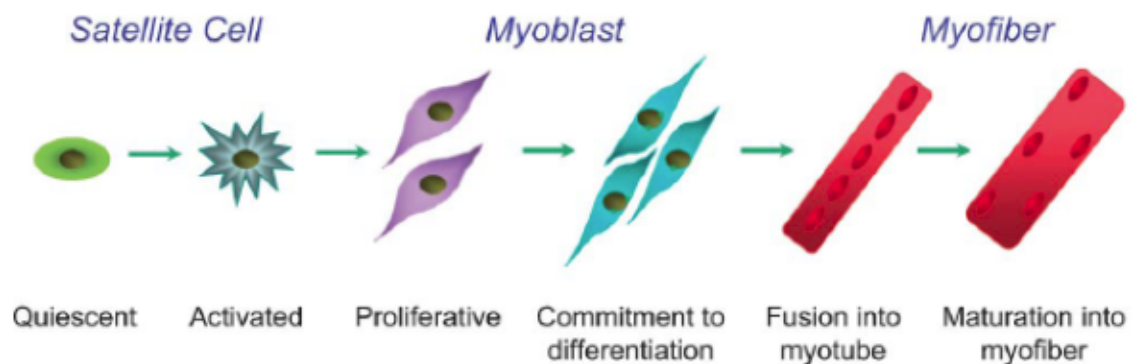


Figure 6. **Schematic representation of satellite cell myogenesis** [12]. Typically satellite cells are quiescent in adult muscle until their activation is invoked by a stimulus such as muscle trauma. Once activated, satellite cells divide to produce satellite cell-derived myoblasts which continue to proliferate. Eventually these cells will commit to differentiation and fuse to form myotubes. These myotubes will then mature into myofibers followed by formation of mature muscle [7].

Satellite cell-derived myoblasts are characterized by expression of myogenic markers including MyoD and Myf5 [83],[84]. After a distinct number of proliferation events, most satellite cells enter the myogenic differentiation program. During this time, they begin to fuse to damaged myofibers or fuse to

one another to produce new myofibers. This stage is initiated upon expression of other myogenic markers including Myogenin and Myf6 (also known as Mrf4) [84].

A final defining characteristic of satellite cells is their remarkable ability to self-renew. This occurs via two mechanisms: asymmetric and symmetric cell division. During asymmetric cell division, one parental stem cell gives rise to two functionally different daughter cells. One of these cells is a stem cell while the other is destined for differentiation. Conversely, during symmetric cell division, one parental stem cell divides into two identical daughter stem cells. Either division process results in maintenance of the stem cell pool [77]. This division potential of satellite cells results in a heterogeneous population [7]. Literature suggests that these different populations of satellite cells exhibit different characteristics. It has been demonstrated that irradiation prevents muscle growth and maintenance due to ablation of most satellite cells [85]. However, a subpopulation of myogenic precursor cells survives and are still able to be recruited to regenerate muscle following a substantial injury [86]. Additionally, with respect to the heterogeneity of satellite cells, studies have revealed the presence of two different satellite cell populations distinct from one another in terms of their mitotic rates. These studies were done using BrdU (bromodeoxyuridine) labeling and they report that ~80% of satellite cells readily enter the cell cycle while the remaining 20% of satellite cells take a lot longer to enter the cell cycle [82]. These researchers propose that this lower population of cells remain in the quiescent state at the beginning of muscle growth/regeneration and only enter the proliferation phase in response for

extensive muscle growth/regeneration. This subpopulation of cells as well as satellite cells in general are overlooked in literature as a potential source of HO. These cells however, as discussed above, have the same potential to be induced into a bone lineage as MSCs and they are already present within the muscle compartment.

2.1.6 Ascorbic acid as an alternative potential prophylactic treatment for heterotopic ossification

Ascorbic acid, also known as vitamin C or ascorbate, is an essential dietary nutrient which is required for various biological functions. Notably, it is required for the biosynthesis of collagen. Under physiological conditions ascorbic acid will hydroxylate proline and lysine residues. This allows for proper intracellular folding of pro-collagen for export and deposition as mature collagen [14]. Additionally, it is involved in the synthesis of carnitine, tyrosine, and various neurotransmitters including norepinephrine and serotonin [87], [88]. Ascorbic acid is also important in regulating iron uptake. It reduces ferric Fe^{3+} to ferrous Fe^{2+} ions. This promotes dietary non-haem iron absorption from the gastrointestinal tract, and also stabilizes iron-binding proteins. Unlike most mammals, humans are not capable of naturally synthesizing ascorbic acid. This is due to a series of mutations of the gene which encodes gulonolactone oxidase. This enzyme functions to catalyze the last enzymatic step in ascorbate synthesis [89]. Due to this inability to synthesize ascorbic acid, humans must obtain the nutrient from their diet. The current recommended dietary allowance (RDA) for ascorbic acid for adult women is 75mg/day and 90mg/day for men [90]. Ascorbic acid can be

found in fresh fruits and vegetables including oranges, lemons, grapefruit, watermelon, strawberries, mango, pineapple, cherries and others. This intake, if maintained, results in 1.2-2.0 g of ascorbic acid within the body. Clinical and epidemiological studies suggest that a dietary intake of 100mg/day of ascorbic acid is associated with reduced incidence of mortality from heart disease, stroke and cancer [15]. Ascorbic acid is generally non-toxic however at high doses (2-6g/day it can cause certain side effects such as gastrointestinal disturbances and diarrhea [91],[21]. These side effects are typically not severe and can be easily reversed by reducing intake of ascorbic acid. There is no consistent data suggesting that very large doses of vitamin C are toxic or that it causes serious adverse health effects in humans [21], [22]. Of all the vitamins, vitamin C has been reported to perhaps have the lowest toxicity [202].

The chemical name of ascorbic acid is 2-oxo-L-threo-hexono-1,4-lactone-2,3-enediol. It exists in two major dietary forms, a reduced form: L-ascorbic acid, and an oxidized form: dehydroascorbic acid (DHA) . The average half-life in an adult human of ascorbic acid is about 10-20 days. It is mainly eliminated through the urine and it's major metabolites are DHA, 2,3-ketogulonic acid and oxalic acid. Ascorbic acid is absorbed in the body via facilitated diffusion and saturable-substrate transport involving various ascorbic acid-specific transporters. Facilitated diffusion is carried out by the GLUT family of transporters (glucose transporters), while active transport depends on the sodium vitamin C transporters (SVCT) [14].

In both human and animal tissues, the highest concentrations of ascorbate are in the adrenal and pituitary glands [102]. Millimolar levels are found in eye tissues [103] which may function to protect the eye tissues from solar radiation damage. Plasma concentrations of ascorbic acid are generally around 50-100 μ M in humans when vitamin C is ingested on a regular basis in an individual's diet. Even with supplementation approaching maximally tolerated doses, ascorbic acid plasma concentrations are always <250 μ M and frequently <150 μ M [104]. In contrast, if ascorbic acid is administered via an IV, pharmacologic concentrations reach about 25-30mM [104]. Other clinical data demonstrate that when ascorbic acid is given orally to patients, at doses of 200mg, plasma concentrations are only approximately 80 μ M. Even if dose is increased to a maximum oral dose of 3g daily, peak plasma values do not exceed ~220 μ M [105], [106]. Conversely, when ascorbic acid is administered intravenously, millimolar concentrations can be achieved. An infusion of 10g of ascorbic acid in cancer patients results in plasma concentrations of 1mM to 5mM [107], [108]. Therefore, only intravenous administration of ascorbate can result in such high plasma levels, (pharmacological levels).

The concentration of ascorbic acid in the plasma and the tissue are related to one another and are dependent on intake. At daily intakes of between 100mg-140mg/day ascorbic acid concentrations are saturated between blood cells and tissue [87]. Studies by Levine and colleagues [109],[110] report intracellular concentrations of ascorbic acid for both males and females. They state that neutrophils, monocytes, and lymphocytes became fully saturated at a

dose of 100 mg daily. Additionally, they report that intracellular concentrations of ascorbic acid reached levels 14-fold higher than in the plasma. For women, after receiving a dose of 60 mg daily, intracellular concentrations reached an average of 1.08 mM in neutrophils [110]. Concentrations in monocytes ranged from 2-3mM after the same dose [110]. After maximum doses of 2500 mg daily concentrations did not reach above 5mM in lymphocytes, monocytes or neutrophils. When men were given a similar 60 mg daily dose of ascorbic acid, intracellular concentrations reached approximately 2-2.5mM in monocytes and did not exceed concentrations much higher than 3mM after being given doses ranging from 500-2500 mg of ascorbic acid daily [109]. Tissue levels of ascorbic acid in skeletal muscle have been reported in the literature to range between 0.17-0.23 mmol/kg of wet tissue or approximately 0.18-0.24 mM (180-240 μ M) in the tissue [92]. Another study performed in C57BL/6 male mice quantified ascorbic acid levels in 14 tissues including skeletal muscle. They report a tissue content of approximately 0.2 μ mol/g of tissue [111]. Studies by Carr and colleagues [112] report a baseline concentration of ascorbic acid in cells as well as in skeletal muscle tissue. They report that baseline concentrations are \sim 60.5nmol/10⁸ cells in mononuclear cells, and \sim 13.7 nmol/10⁸ cells in neutrophils. Concentrations in muscle tissue were reported at an average of \sim 16 nmol/g of skeletal muscle tissue [112]. Ascorbic acid concentrations have also been reported in interstitial fluid. Dabbagh and Frei [113] collected human suction blister interstitial fluid (SBIF) and determined that total ascorbate is present in the fluid at a concentration of approximately 96 μ M. Other studies have demonstrated

that SBIF is a suitable representative of interstitial fluid (IF) in the body [114], [115].

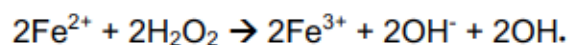
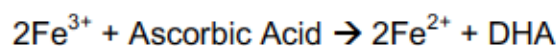
In 1992, a 12-year old patient suffered from MOP, which is an autosomal dominant condition involving severe heterotopic bone formation. The disease progressed quickly resulting in many ossifications in the patient's back and limited arm movement. In 1992 the individual began treatment with 500mg/day ascorbic acid. The treatment was increased over time and symptoms improved significantly. Palhares hypothesized that ascorbic acid helped to stabilize the synthesis of procollagen III controlling this disease [20]. This theory has not been proven and after this point ascorbic acid has not been reviewed again in literature as a potential treatment (prophylactic or other) for HO. Based on what has been previously discussed regarding current prophylactic treatments however, there is an alternative therapy needed other than the use of NSAIDs or radiation therapy to prevent HO. Ascorbic acid may be an alternative prophylactic treatment to these methods based on specific properties of the vitamin discussed below.

It is widely accepted that a triad consisting of glutathione, ascorbic acid, and vitamin E are the central antioxidant defense mechanisms in mammals and insects. Each of these compounds is a major ROS scavenger. It is important to note however that their actions are distinct from ROS scavenging by superoxide dismutase (SOD) and catalase [93]. At physiological or normal concentrations, ascorbic acid functions as a water-soluble antioxidant, scavenging harmful radicals and protecting cells against oxidative damage caused by ROS [15]. Ascorbic acid is able to function effectively as an antioxidant because when it

reduces ROS, it causes formation of a much less harmful ascorbate free radical. This ascorbate free radical is then reduced back to ascorbic acid within the cells by NADH- and NADPH-dependent reductases. Ascorbic acid is thought to be able to counteract inflammation and oxidative damage which are responsible for the initiation and progression of many chronic and acute disease when it functions as an antioxidant. It is also known to help individuals suffering from the common cold. In addition to functioning as an antioxidant, ascorbic acid also functions as a pro-oxidant. Casciari and colleagues [16] concluded that low doses of ascorbic acid result in antioxidant protection to tumour cells, while high doses increased cell death. Pharmacologic or increased doses of ascorbic have been shown to induce toxicity and oxidative stress in pancreatic cancer cells by Du and colleagues [17]. Pharmacologic concentrations of ascorbic acid in the extracellular environment can oxidize to form hydrogen peroxide (H_2O_2). This newly formed H_2O_2 will then diffuse across the cell membrane causing oxidative damage to cellular proteins, lipids, and DNA [17]. This suggests a pro-oxidant role for ascorbic acid at increased concentrations. Therapies that have been designed to increase oxidant stress using methods such as ionizing radiation in combination with pharmacologic ascorbic acid are being investigated as a method to preferentially sensitize tumour cells versus normal cells via metabolic oxidative stress [17],[18]. Ascorbic acid-mediated H_2O_2 formation additionally causes DNA damage via transition metal ions including Fe^{2+} [94]. Buettner and Jurkiewicz [95] suggest that the presence of catalytic metals may be a determining factor if it functions as a pro- or antioxidant. In the absence of

transition metals, ascorbic acid can only function as an antioxidant. If the concentration of these metals increases however, ascorbic acid exhibits pro-oxidant properties and may be dangerous [95].

Transition metals are naturally present in buffer solutions. Trace levels of these metals can pose as a problem for researchers because they may catalyze reactions including oxidation of ascorbate. Ascorbic acid will readily autoxidize transition metals such as copper and iron [96]. Because ascorbic acid is a well-known reducing agent, in the presence of these metals, it will reduce ferric (Fe^{3+}) to ferrous (Fe^{2+}) iron, resulting in pro-oxidant effects [95]. This also occurs with copper. Once this occurs, ascorbic acid will be simultaneously oxidized to ascorbic acid radical ($\text{Asc}^{\cdot-}$) via two consecutive, one-electron oxidations. The produced Fe^{2+} may then react with O_2 in order to produce superoxide radical ($\text{O}_2^{\cdot-}$). H_2O_2 will then be produced as a final product when the superoxide radical reacts with hydrogen ions. The toxicity of ascorbic acid will depend on the oxidation and generation of H_2O_2 . In the absence of transition metals, oxidation of ascorbic acid is fairly slow. Thus the rate of oxidation is accelerated by catalytic metals [95]. The reaction in which ascorbic acid generates highly reactive free radicals in the presence of transition metals is known as the Fenton reaction [97]:



These hydroxyl radicals interact with DNA and cause damage by breaking the phosphodiester backbone. As well they will modify DNA bases [98].

Researchers believe that ascorbic acid can be used to prevent cancer by neutralizing free radicals before they can damage DNA and initiate tumour growth. Additionally, ascorbic acid may exhibit its pro-oxidant functions and help the body's free radicals to destroy tumours in their early stages [19]. As previously discussed, ascorbic acid oxidizes to produce H_2O_2 . This causes oxidative stress in the cell. Oxidative stress can be defined as a disturbance in the balance between the production of ROS and antioxidant defenses [100]. Pharmacological ascorbate has been proposed as a pro-drug for the delivery of H_2O_2 to tumours based on this principle [101], [17], [27]. Based on these properties, at the appropriate dose, ascorbic acid could potentially function to prevent HO by increasing oxidative stress in a similar mechanism as radiation therapy, without the harmful risks or side-effects. Treatment doses of ascorbic acid would ideally prevent osteogenesis of bone-forming cells and either promote or maintain myogenic differentiation of cells involved in muscle repair. Literature has demonstrated that ascorbic acid will contribute to osteogenic differentiation of MSCs derived from bone-marrow sources by increasing the secretion of collagen type 1 into the extracellular matrix. This is because ascorbic acid acts as a cofactor for certain enzymes responsible for hydroxylating proline and lysine in pro-collagen [99]. Previous literature has demonstrated that a dose of $50\mu M$ of ascorbic acid will contribute to osteogenesis of MSCs [24]. As such the treatment doses of ascorbic acid investigated in this study ($50\mu M$ to $500\mu M$) should not interfere with natural bone repair at the injury site. In summary, the use of ascorbic acid in place of radiation therapy or NSAIDs would eliminate harmful

risk factors involved with these treatments. In addition, it would be easier and faster to administer to patients, as well as inexpensive.

2.1.7 In vivo models of heterotopic ossification

Many animal models of HO exist, however these models induce heterotopic bone formation with the use of exogenous agents such as BMPs or bone matrix [116], [117],[118],[119]. Other non-physiological models [120],[117],[121] do not produce relevant patterns of injury which factor in important elements associated with a combat-injury. Therefore, these models do not adequately represent the mechanism of injury and pathologic process involved with formation of heterotopic bone in high-energy blast amputees. In order to fully understand this condition, an animal model which effectively and reliable reproduces pathological formation of HO after a high-energy extremity blast amputation is needed. In 2011, studies by Tannous and colleagues [122] resulted in a reproducible model for HO in the residual limbs of blast-amputated rats without addition of exogenous osteogenic stimulus. This study was said to be the first animal extremity blast model produced to cause spontaneous formation of heterotopic bone. All four of their surviving hind limb amputees sustained amputation at a below-knee level and exhibited Type A (periosteal growth) bone growth. Three of those four also exhibited adjacent sections of heterotopic bone (Type B: non-contiguous growth) within the area of soft tissue injury in the stump. Additionally, one of the five forelimb amputation stumps exhibited both Types A and B HO. However, in 2015, Polfer and colleagues [123] stated that there was no animal model in which to evaluate the underlying

physiological processes which lead to HO formation due to combat-related and blast wounds. They suggested that the model proposed by Tannous and colleagues [122], which involved a traumatic amputation using a high explosive, failed to involve total body exposure. The portion of the rat proximal to the knee (or elbow) was shielded from the blast wave, limiting the animals total body exposure to the blast overexposure (BOP). Additionally, these researchers did not quantify the degree or consistency of the BOP delivered to the animal. These factors may contribute to the local and/or systemic inflammatory response. Polfer *et al.* [123], therefore used a variety of techniques to produce HO in the animals including: blast overpressure (BOP), extremity trauma coupled with amputation, and a combination of all three treatments. They conclude that HO formed in all 20 animals exposed to BOP, thigh crush, femoral fracture, and transfemoral amputation through the injured region. The model that they proposed involves highly controlled interventions and is likely a more reproducible and more widely accepted model than the one proposed by Tannous *et al.* in 2011.

2.1.8 *In vitro* induction of heterotopic ossification

For nearly half a century, it has been known that demineralized bone can induce *de novo* bone formation [124]. As such, the therapeutic potential for bone formation induced by this demineralized bone or its extracts has also been recognized [124], [125], [126]. Later studies identified the presence of bone-forming factors in demineralized bone which were termed BMPs [127], [128], [129]. Therefore, it is logical that the most common method used to study HO, *in vitro*, reported in the literature is through osteogenic induction of myoblasts using

a specific protein known as BMP-2. The use of BMPs to induce bone formation in these tissues is well established. These BMPs are important in terms of skeletal development and bone formation [130], [131], [132]. It is important to note that BMP is not a single protein, rather there are many related yet distinct proteins classified as BMPs. In total, there are 14 BMPs that have been identified [133],[13]. These proteins will bind different BMP receptor classes which exist as homodimers. This leads to phosphorylation of Smad proteins. Once phosphorylated, these Smad proteins can act as transcription factors on many target genes including a master regulator of osteogenesis, *Runx2* [134]. All BMPs are related to each other and can be classified into multiple subclasses of the transforming growth factor- β (TGF- β) family, except BMP-1 which is distinct in terms of structure and belongs to the family of metalloproteinases [128]. Certain BMPs including; BMP-2, BMP-4, BMP-6, BMP-7, BMP-9, BMP-12, and BMP-13 have all been shown to induce differentiation of MSCs, while BMP-3 has been shown to induce MSC proliferation. HO *in vivo* has been shown in literature to be induced by BMP-2, BMP-4 and BMP-7 [128], [116] and even more studies have demonstrated these BMPs as well as a few others induce bone formation in general *in vivo* [135], [136], [137], [138], [139], [140], [141], [142], [143]. This is important because it shows that there is original BMP activity involved.

C2C12 cells are murine myoblasts established from cells of the regenerating thigh muscles in C3H mice [144]. C2C12 cells are widely used in the literature to study myogenesis *in vitro* because as they grow they express MyoD, followed by differentiation into myocytes and eventually multinucleated

myotubes. These myotubes express proteins involved with contraction such as myosin heavy chain and troponin T. In addition to their use in studying myogenesis, C2C12 cells are considered a pre-osteoblastic cell line [13]. As such they are commonly used to study HO *in vitro*. When treated with BMP-2, expression of the myogenic phenotype in C2C12 cells is inhibited, and these cells remain as mononuclear cells. Additionally, BMP-2 causes osteogenic processes in C2C12 cells such as elevated levels of ALP and the expression of osteocalcin [23], [13]. C2C12 cells that have been transduced with BMP-2, BMP-6, BMP-7 or BMP-9 induced formation of bone within the quadriceps muscle of nude mice *in vivo* [133].

Studies by Cheng *et al.* [13] demonstrate that BMP-2, 6, and 9 exhibited the greatest ability to induce both early and late osteogenic markers and matrix mineralization in osteoblastic progenitor cell lines. These data were deduced by measuring markers of differentiation including ALP activity, osteocalcin expression and matrix mineralization. They have developed a hierarchal model of BMPs based on their osteogenic activity [13]:

Osteogenic Hierarchy of BMPs

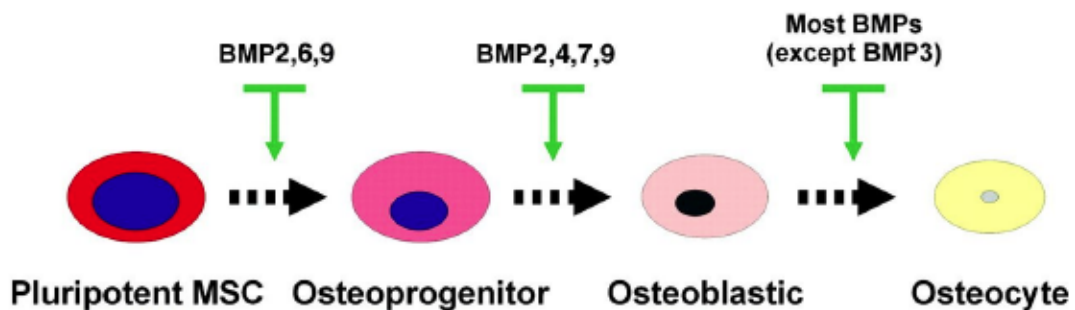


Figure 7. **Osteogenic hierarchy of bone-morphogenetic proteins (BMPs)** [12]. A hierarchal model of BMPs is illustrated based on the osteogenic activities

of each. These findings were determined based on studies involving pluripotent mesenchymal progenitor C3H10T1/2 cells, pre-osteoblastic C2C12 cells, and osteoblastic TE-85 cells.

Based on this model and corresponding studies, it is clear that BMP-2, 6 and 9 are the most potent agents to induce osteogenic differentiation in mesenchymal progenitor cells. The signal transduction processes involved with BMP induced bone formation are described in Figure 8.

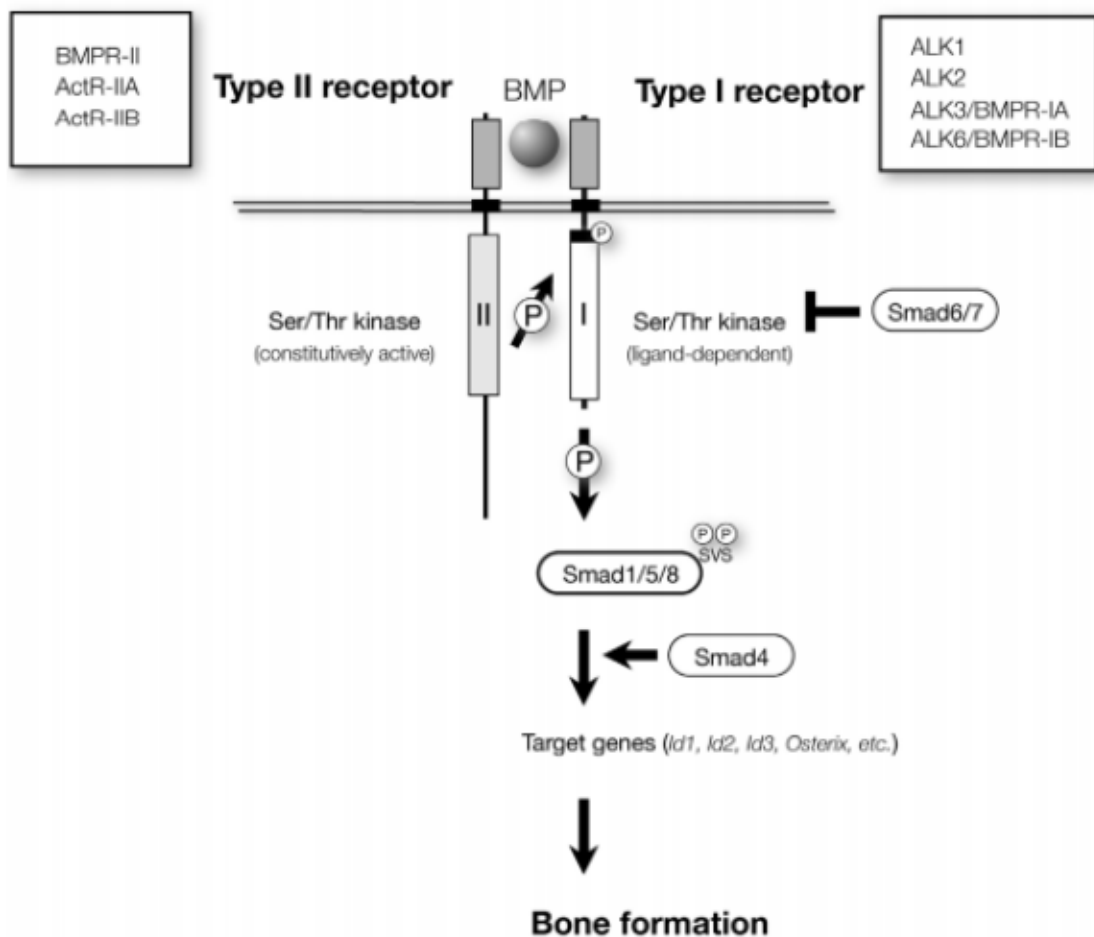


Figure 8. **Signal transduction of bone-morphogenetic protein (BMP)-induced bone formation** [145]. BMPs bind to type I and type II active serine / threonine kinase receptors. The type II receptor phosphorylates the type I receptor which then activates it. Once activated the type I receptor

phosphorylates Smad 1/5/8, which results in the formation of complexes with Smad4. This complex regulates transcription of target genes to induce bone formation [145].

Just as with myogenic differentiation, there are markers of differentiation which exist to signify osteogenesis. Osteocalcin for example is a well-known marker of bone formation. It is expressed in the later stages of osteogenic differentiation, defining a more mature osteoblastic phenotype within the osteocyte [146]. The actual function of osteocalcin is unknown, however literature suggests that it may have a role in regulation of osteoblast function. Ducy and colleagues [147] showed that the 'osteocalcin knockout' mouse demonstrated no abnormal phenotype until 6 months of age. At this time there was also increased bone formation observed. Osteopontin in contrast is expressed in developing bone cells during early stages of osteogenesis, prior to mineralization or osteocalcin expression. As such osteopontin is often used as an early marker of osteogenic differentiation in terms of identifying an osteoprogenitor cell [148]. Osteogenic differentiation markers are important in terms of determining presence of osteogenesis whether it is natural or ectopic bone that is being formed. As such, they can technically be classified as contributing factors to HO based on their presence. Another contributing factor to HO is inflammation. Inflammatory cells including lymphocytes, macrophages, and mast cells are located in early HO lesions within skeletal muscle and connective tissue. The presence of inflammatory cells is associated with damage to skeletal muscle cells and tissue hypoxia. These factors are capable of triggering the proliferation of undifferentiated MSCs, suggesting that inflammatory and skeletogenic

signaling pathways are critical in HO formation [149]. A study by Kaplan *et al.* [150] demonstrated that inflammatory cells of the hematopoietic lineage trigger HO in fibrodysplasia ossificans progressiva (disorder) as well as in BMP-induced HO. Individuals suffering from fibrodysplasia ossificans progressive develop ectopic bone due to a genetic dysregulation of BMP signaling in the presence of inflammatory triggers [149]. Similarly, Lounev *et al.* [149] showed that muscle injury and associated inflammatory changes are sufficient to trigger fibrodysplasia ossificans progressiva-like HO in a setting of chronically stimulated BMP activity. Other studies have demonstrated that macrophages are recruited to an early injury site following muscle trauma in Nse-BMP4 mice. These data suggest that macrophages are involved in the induction of injury-induced HO in these mice [151]. Although still under extensive investigation, HO is poorly understood. Understanding the pathogenesis and mechanisms involved with the condition are the first steps in developing appropriate therapies and therefore there should not be any factors ignored.

Chapter 3. Experimental Design

3.1 Limitations, Basic Assumptions and Delimitations

3.1.1 Limitations and Basic Assumptions

The following limitations and basic assumptions are inherent to the project and set by the investigator:

Immortalized cell lines commonly used for studying skeletal muscle physiology include L6 and C2C12 cells, which are derived from rat and mouse origin [152]. Under the appropriate conditions, these cells can be terminally differentiated into skeletal muscle myotubes and therefore used as a model to study muscle physiology. A better model involves the use of primary cells isolated directly from an animal or host as they retain phenotypic traits of the donor [153]. Quiescent satellite cells (MPCs) can be isolated from numerous tissues including rat skeletal muscle and, when they are cultured in appropriate conditions, they can be stimulated to re-enter the cell cycle and proliferate followed by terminal differentiation resulting in myotube formation. The first limitation to this study is that Sprague-Dawley rats are being used as the only source of cells. Different strains are not being tested. There are additional limitations associated with primary cell culture as well. Firstly, unlike immortalized cell lines, which have indefinite myogenic potential, once primary cells such as MPCs have been isolated they are fated for senescence and after a relatively short period in culture will stop proliferating. It has been shown in the literature that passaging of rat primary cells results in decreases in myogenic potential.

Machida and colleagues demonstrate that after three passages, the percentage of cells positive for myogenic markers desmin, MyoD and Pax7 was reduced to 55% [154]. In order to maintain confluent cultures or differentiated cells in differentiation conditions there is a limited time MPCs can last before they begin to detach from the plates. As such, calcification assays such as alizarin red stain (ARS) which require a longer period of time for calcification cannot be performed to their full potential.

Although a primary cell culture model allows for accurate study of muscle cell structure and function as well as many metabolic abnormalities which exist *in vivo*, certain environmental influences on the muscle are eliminated in a cell culture model. These include for example, circulating hormones, adipokines and other bioactive factors. As such, when investigating ascorbic effects on myogenic potential of MPCs, all factors are not considered. Additionally, osteogenesis is being induced in a specific and non-physiological manner. There are many other factors *in vivo* which are not considered during *in vitro* studies such as growth factors or other cells in the environment which can influence osteogenesis. One example of such a growth factor is TGF- β [155]. Other examples include inflammatory cells, macrophages, which are also recruited to the tissue injury site [149], [151]. The best way to study HO would be in a human model, however it is not feasible to do studies on individuals directly after a military induced blast-injury. Human cells can be collected from individuals after surgery or other trauma however the *in vitro* studies still must be performed. The methods described to study HO include use of osteoinductive factors such as BMPs and

osteogenic medium, however these methods are only a model to study osteogenic potential and not HO. In order to accurately replicate HO, *in vivo* experiments would need to be conducted, however again, HO is not a guaranteed reproducible condition. These proposed *in vivo* experiments would be useful to determine if ascorbic acid would be a viable option for clinical trials.

Finally, in order to further attempt to determine which specific cells are responsible for causing HO, cell migration assays would need to be conducted. This is another limiting factor as resources are unavailable for such an experiment in terms of the length of this study. Further studies would need to be conducted in the future to obtain this information.

3.1.2. Delimitations

The following delimitations are inherent to the project and set by the investigator:

Although experiments with primary cells are preferred, there are certain delimitations which result from their use. Firstly, upon collection of cells from the animals (see section 3.2) it is assumed that these animals are healthy and pathogen free. Health reports of these animals are provided from the vendor so it needs to be assumed that these animals are healthy, however disease can be acquired.

Secondly, the MPCs isolated from Sprague-Dawley rats will differ between animals. An experiment using a cell line results in exact copies of one another, while primary cells directly taken from an animal will vary between animals. One way to help minimize this error is to repeat all experiments in each different

animal, rather than doing all experiments on one animal or doing different experiments between animals.

3.2 Animals and Cells

All rats used to obtain primary satellite cells were male, Sprague Dawley strain, age: 73-77 days, weight: 326-350 grams (obtained from Charles River Laboratories). Rats were housed under controlled temperature (18-20°C), humidity (40-70%), decibel level (<70dB), and lighting (12 hours of light; 12 hours of dark) ad libitum. All animal experiments were performed in accordance with the institutional animal care committee guidelines at Lakehead University. Figure 9 below describes an overview of the processes involved with dissecting, isolating, freezing and growing primary cells. All experiments were performed on passage 1 cells.

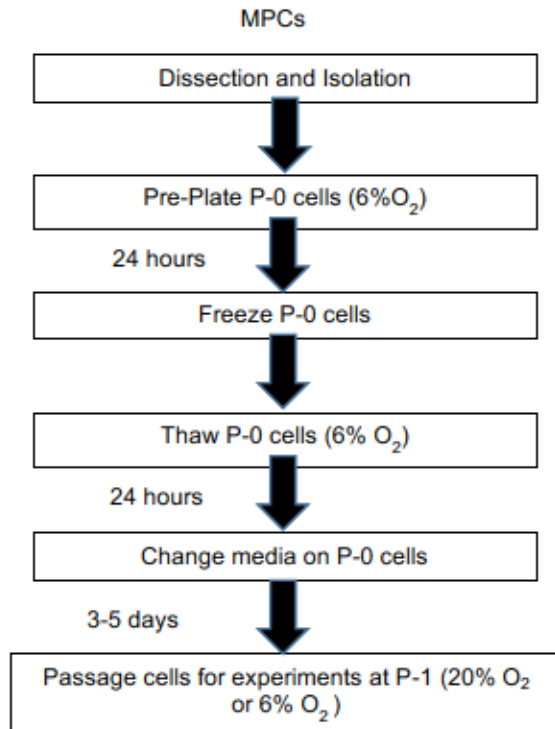


Figure 9. **Schematic representation of isolation and growth of MPCs** . Muscle precursor cells (MPCs) are first dissected and isolated from the animal followed by plating and incubation in 6% oxygen (O_2) at $37^\circ C$. At this stage plates are not coated with Matrigel (MG) in order to purify the population of cells. They can be frozen at $-80^\circ C$ followed by long-term storage in liquid nitrogen long term 24 hours later. Cells are thawed in 6% O_2 on MG-coated dishes to allow adhesion of cells. Approximately 24 hours after initial thawing media must be changed to remove dimethyl sulfoxide (DMSO) incorporated during freezing. Cells are then passaged for experiments at P-1 (passage-1) 3-5 days later at the appropriate O_2 level.

3.1.1 Isolation of MPCs as per Lees-Satellite Cell Isolation Lab Protocol

Animals were anaesthetized using isoflurane (USP – PPC, Richmond Hill, ON, cat # CP046V2) followed by shaving of the hind limbs. Shaved hind limbs of the animal were then rinsed with 70% ETOH (Histoprep 70% EA, 70% denatured ethyl alcohol – Fisher Scientific, Fair Lawn, NJ, cat # HC1000) in order to clean the region. Using the process of exsanguination, the heart was removed from the animal. Next, the gastrocnemius, plantaris, and soleus muscles were removed

and placed into a 100mm culture dish with DPBS (HyClone™Dulbecco's Phosphate Buffered Saline, Solution – Thermo Scientific, cat # SH30028.02). Excess tissue and fascia were removed from the muscle using haemostats and forceps then the trimmed muscles were placed into fresh DPBS. The muscle tissues were then minced in one pile for 10 minutes on piece of sterile filter paper placed on a flame sterile metal tuna can (the surface of this tuna can was soaked in ethanol and the can was placed on a piece of ethanol-soaked paper towel. Before mincing, the ethanol was poured off and the surface was allowed to dry). Throughout the process, it was important to frequently wet the filter paper with DPBS. The minced muscle was then transferred to a 50ml conical tube, topped up to 20 ml DPBS and triturated with a 10ml serological pipette. Next samples were centrifuged at 1500xg for 5 minutes. During this time the Protease Buffer (sterile DPBS + 1.25mg/ml protease type XIV (Bacterial, from *Streptomyces griseus*, contains calcium acetate, Sigma-Aldrich, St. Louis, MO, cat # P5147-5G)) was prepared in an autoclaved beaker with stir bar and sterile filter (75ml/rat). After centrifugation, the supernatant was discarded by decanting and enough protease buffer was added to make up to a final volume of 30 ml, followed by trituration. Next, half (15 ml) of the protease/muscle mixture was transferred into a fresh 50ml conical tube and brought up to final volume of ~35-40ml protease buffer and inverted 10x. Tubes were then incubated in a 37°C water bath for 1 hour while inverting 10x every 15 minutes. After the 1-hour incubation, tubes were centrifuged at 1500xg for 5 minutes and the supernatants were discarded. Pre-warmed (37°C) DPBS was then added up to 20 ml to each

tube followed by trituration approximately 10x. Samples were then centrifuged at 500xg for 10 minutes. After centrifugation, the supernatants (containing cells) were poured into new sterile 50ml tubes and pre-warmed DPBS was added up to 15 ml to the pellet followed by trituration 5x. Next samples were centrifuged at 500xg for 8 minutes and supernatants were carefully poured off into the 50 ml tubes containing cells from above. At this point, warm DPBS was added up to 15 ml to the pellet and triturated 5x. After trituration, samples were centrifuged at 500xg for 1 minute. Next, the supernatants were poured into the 50 ml tubes containing cells from above. Supernatants were then strained through a 100 μ m cell strainer using a 50-ml serological pipette (the pipette was pressed directly to the strainer and gently forced through mesh. The first tube was strained into a fresh 50ml tube then the strainer was moved to a dirty 50 ml tube and the second tube was strained into that. It was important to ensure that both tubes had equal volumes of strained supernatant). Samples were then centrifuged at 1500xg for 5 minutes to pellet the cells. The resulting supernatant was decanted into a plastic beaker and 2 ml of pre-warmed PPM (pre-plate media): DMEM HG (Dulbecco's Modified Eagle's Medium: Thermo scientific – cat# SH30022.01) + 10% Donor Equine Serum (Thermo scientific – cat#SH30074.03) + 10 μ g/ml Gentamycin (Gentamycin Sulfate Solution – MP Biomedicals, Solon, OH, cat # 105030) + 100U/ml Penicillin + 100 μ g/ml Streptomycin (HyClone® Penicillin-Streptomycin Solution, 0.1 μ m sterile filtered – Thermo Scientific, Logan, Utah, cat # SV30010) was added to each cell pellet. Cells were then gently triturated 5x. After sufficient trituration, the pellets were combined into one 50ml tube followed by the addition

of 16ml PPM for a total of 20ml. Cells were then gently mixed and plated onto a 150mm tissue culture dish which had been pre-coated with Matrigel (MG: BD Matrigel™ Basement Membrane Matrix, High Concentration – BD Biosciences, Bedford, MA, cat # 354248) diluted to a 0.1mg/ml concentration in serum-free Ham's F-10 culture media for 60 minutes at 37°C. Plates were coated with MG at this stage in order to allow adhesion of the MPCs to the plastic culture dish. The 150mm dish containing cells was then incubated at 37°C for 24 hours in 6% O₂.

3.1.2 Freezing MPCs

After 24 hours, the 20 ml of PPM was removed from the 150 mm culture dish and added to a fresh 50 ml conical tube containing the non-adherent MPCs. Using 8 ml of warm PPM, the plate was washed down 5x, then all of the solution was taken up and added to the 50 ml tube from above. The tube was then centrifuged at 1500xg for 5 minutes (during this time a 20% DMSO (Dimethyl Sulfoxide, BioReagent for molecular biology – Sigma-Aldrich, St. Louis, MO, cat # D8418) solution was prepared by diluting in growth medium)). After centrifugation, the supernatant was poured off into old 150 mm plate and the pellet was re-suspend in 2 ml of warm growth medium (GM): Ham's F-10 20% FBS (Ham's F-10 from Thermo scientific – cat #SH30396.03, Fetal bovine serum (FBS) from Thermo Scientific – cat# SH30396.03) + 100U/ml Penicillin + 100ug/ml Streptomycin + 10µg/ml Gentamycin). Next, 2 ml of 20% DMSO solution was added drop-wise to the 2 ml cell suspension while swirling the tube (total of 4 ml of 10% DMSO/cell solution). Finally, 1 ml of the DMSO/cell solution was aliquoted into each of 4 fresh, labelled cryovials and stored at -80°C for 24

hours (suspended in cotton balls in Styrofoam box). After 24 hours the cells were moved to long-term storage in the vapour phase of liquid nitrogen.

3.1.3. Thawing and growing MPCs

Prior to beginning to thaw cells, an appropriate amount of MG from -20°C was thawed in a beaker with RT water. Approximately 1 hour prior to thawing cells, a 150 mm tissue-culture dish was coated with a 0.1mg/ml concentration of MG + media (20% FBS Ham's F-10 media: Ham's F-10 media: Hyclone Nutrient Mixture – Ham's F-10, Thermo Scientific – cat#SH30025.01) mixture and incubated for 1 hour at 37°C in 6% O_2 . After the 1-hour pre-coating cells were obtained from liquid nitrogen and, after cracking the seal to allow for air to escape, the cells were thawed in a 37°C water bath until only a small ice chunk remained. Next, the thawed 1 ml cell suspension was transferred to a fresh, sterile 50 ml tube. At this point, drop-wise, while swirling the tube, 9ml of warm GM + MG from the 150mm plate (see above) was added very slowly to the 50 ml tube (this allowed osmolalities to equilibrate so cells do not burst). The next step was to obtain all GM/MG + cell suspension from the 50 ml tube and add it back to the 150 mm culture dish. The plate was then tilted back and forth and side to side to ensure even distribution of the cells and incubated for 24 hours at 37°C in 6% O_2 . The following day, once the cells had adhered, the media containing DMSO was aspirated out and, after a DPBS wash, changed to fresh 20% FBS Ham's F-10 media. From this point on, the media was changed on the cells every 2 days to fresh 20% FBS Hams F-10 until the cells reached ~80% confluency and were ready to passage.

3.1.4 Cell Passaging

Once cells had reached ~80% confluency, they were passaged and seeded for appropriate experiments according to Appendix 6.1 (Cell Splitting Protocol: Lees Lab). For MPCs, plates and flasks were first coated with a small volume of 0.1mg/ml MG. Cells were detached from the flasks or plates using a trypsin-based cell dissociation enzyme solution such as TrpLE™Express (1x, no phenol red, Invitrogen – Gibco, Thermo Scientific, Logan, Utah, cat# 12604-054) or Trypsin (Trypsin 0.25% (1X) Solution with 2.5g Porcine Trypsin, without calcium or magnesium, with EDTA – Thermo Scientific, Logan, Utah, cat # SH30042). All MPCs were centrifuged at 150xg for 7 minutes. After seeding, all proliferation experiment plates were placed at 37°C in 6% O₂ while all differentiation experiment plates were placed at 37°C in 20% O₂. Refer to Appendix 6.5 for specific media and solution volumes.

3.3 Assays and Experimental Design

Table 1 below describes an overview of the experimental design for this project. Rat primary MPCs were treated according to the physiological process being tested.

Cells	Physiological Process	Media	Treatment	Assays
MPCs	Osteogenesis	GM	-0, 10, 100ng/ml BMP-2 -0, 100 μ M Ascorbic Acid -0, 1mM TEMPOL -0, 200U PEG-Cat	-Alizarin Red Stain Quantification -Alkaline Phosphatase Activity
MPCs	Proliferation	GM	-0, 50, 100, 200, 500 μ M Ascorbic Acid	-BrdU Incorporation
MPCs	Myogenic Differentiation	GM	-0, 50, 100, 200, 500 μ M Ascorbic Acid	-Dual-Luciferase Reporter Assay -Western Blot
MPCs	Myogenic Differentiation	DM	-0, 50, 100, 200, 500 μ M Ascorbic Acid	-Dual-Luciferase Reporter Assay -Western Blot

Table 1. **Overall experimental design based on the physiological process being tested.** After treatments were applied to muscle precursor cells (MPCs), each physiological process was tested with various assays outlined in the figure above. GM: growth medium, DM: differentiation medium, AA: ascorbic acid.

3.3.1 Assessing purity of MPC population: Pax7 expression in MPCs

In order to assess the purity of the isolated MPCs, a well-known satellite cell marker Pax7 was used. First cells had to be cultured and fixed followed by introduction of the Pax7 antibody and flow cytometry analysis to assess if the isolated MPCs were positive for the Pax7 marker.

3.3.1.1 Fixing MPCs

Cells were passaged in a T25 flask, seeded at 250K cells and once they had reached ~70-80% confluency (refer to section 3.1.6 for cell passaging protocol), they were fixed by aspirating the media from the T25 flask and washing with DPBS. Next, 1 ml of a trypsin-based enzyme dissociation solution was added in order to detach the cells from the flask. This trypsin-based enzyme dissociation solution was then neutralized with 2 ml of warm GM. Samples were centrifuged at 150xg for 7 minutes (in 15ml tubes) then the supernatant was decanted and the pellet re-suspended in 1 ml of pre-warmed GM. Cells were then counted as per Appendix 6.1. Next the cells were centrifuged a second time at 150xg for 7 minutes and the pellet was re-suspended in 200 µl of DPBS. Once the pellet was fully re-suspended in DPBS, 4 ml of ice-cold methanol (certified ACS, acetone free, absolute – Fisher Scientific, Fair Lawn, NJ, cat # A412P-4) was added drop-wise to the cells while vortexing on a low speed. The fixed cells were then stored at 4°C until flow cytometry could be performed.

3.3.1.3 Antibodies and flow cytometry preparation

Samples from section 3.3.1.2 were removed from 4°C and centrifuged at 200xg for 15 minutes at 22°C. The supernatants were then poured off and the cells were re-suspended in 1 ml of wash buffer (1% BSA/DPBS. BSA: Bovine serum albumin, fraction V, RIA and ELISA grade, CAL BIOCHEM –cat #126593). After re-suspension, the 1 ml cell suspension was added to a fresh, labelled 1.5 ml Eppendorf tube (coated prior for at least 20 minutes with 1 ml of wash buffer). Samples were then centrifuged at 400xg for 5 minutes at 22°C (with the tubes

facing the same way to know the pellet location). After centrifugation, the supernatant was carefully removed using a pipette and 100 μ l of primary antibody (Anti-PAX7 antibody [EPR3353], Abcam - #92317, Cambridge, MA, USA), diluted 1/200 in blocking buffer (3% BSA/DPBS) was added to the cells. Note that the antibody was first given a flick and a zip-spin to prevent any loss of reagent in the cap. Tubes were then allowed to sit in the dark at room temperature (RT) for 30 minutes. During this time, the secondary antibody (Goat Anti-Rabbit IgG H&L [Alexa Fluor®488], Abcam - #150081, Cambridge, MA, USA) was prepared at a dilution of 1/2000 in blocking buffer (3% BSA/DPBS) and stored at 4°C. After 30 minutes, samples were centrifuged at 400xg for 5 minutes at 22°C and re-suspend in 250 μ l of wash buffer. This was repeated for a total of 3 washes (carefully so as not to lose the pellet). On the final wash, the cells were re-suspended in 100 μ l of secondary antibody (prepared above). The cells were then incubated in the dark for 30 minutes at RT. Following the 30-minute incubation, cells were centrifuged at 400xg for 5 minutes at 22°C and re-suspended in 250 μ l of wash buffer. This step was repeated for a total of 3 washes and on the final wash the cells were re-suspended in 1 ml of wash buffer and transferred to fresh 5 ml polystyrene round-bottom tubes for flow cytometry.

3.3.1.4 Flow cytometry (computer set-up and parameters)

Flow cytometry was performed using the BD CellQuest Pro™ software on a BD FACS Calibur™ Flow Cytometer (BD Biosciences, San Jose, CA USA, model #342975). A pre-existing template was utilized that was suitable for

MPCs. The voltage was set to E-1, with a gain set to 5.08. The mode was linear and the data file was set to G1=R1 with 'no gait'.

3.3.2 Experimental overview of specific aim #1

The objective of specific aim #1 was to investigate the potential of MPCs to be induced into a bone lineage. In order to do this, cultured MPCs were first treated with various doses of BMP-2 including 0, 10, and 100ng/ml of BMP-2. Once cells were treated appropriately a series of assays including the ALP activity assay and ARS quantification were performed in order to assess osteogenesis of MPCs.

3.3.2.1 *BMP-2 Induced Osteogenesis*

In order to induce osteogenesis in MPCs, cells were seeded at a density of 50k cells in 12-well plates and placed in 20% O₂. After cells reached 90-95% confluency (began to swirl but no myotube formation) cells were treated with a dose of 0, 10, or 100ng/ml BMP-2 (BMP-2 was added to fresh media first then the media was changed in each well). Cells were then differentiated for a total of 3 days for the ALP activity assay and 5 days for the ARS quantification. The media and BMP-2 treatment was changed every second day. After 3 days of differentiation the cells were lysed for ALP. After 4 days, cells were fixed for ARS.

3.3.2.2 *Lysis of cells to determine alkaline phosphatase activity*

After 3 days of BMP-2 induced osteogenic differentiation, MPCs from section 3.3.5.2 were lysed for ALP. First, plates were washed 2x with DPBS then 250 µl (for a 12-well plate) of lysis buffer (LB: 0.1M Tris (TRIS Crystallized Free

Base, Molecular Biology Grade – Fisher Scientific, Fair Lawn, NJ, cat # BP152-1) + 0.1% Triton®-X-100 (Electrophoresis Grade – Fisher Scientific, Fair Lawn, NJ, cat# BP151-500)) was added to each well. Once the lysis buffer was added to the wells the plates were sealed with parafilm and immediately frozen at -80°C until the ALP assay could be performed.

3.3.2.3 Alkaline phosphatase activity assay

In order to determine ALP activity, the frozen plates from section 3.3.2.2 were first thawed on *The Belly Dancer* (Stovall Life Science Incorporated, Greensboro NC USA, model# BDRAA115S) on a low-medium speed. During this time the assay reagents were prepared. The SIGMA FAST Reagent was prepared by dissolving 1 tablet of Tris Buffer (SIGMA FAST™ TRIS BUFFER – Sigma-Aldrich, USA, cat # T8915) and 1 tablet of pNPP reagent (SIGMA FAST™pNPP – Sigma-Aldrich, USA, cat # N9389) from the SIGMA FAST™ kit (SIGMA FAST™ p-Nitrophenyl phosphate tablets – Sigma-Aldrich, USA, cat# N1891-50SET) in 5ml of DW. Once the plate had thawed completely, the lysate in each well was triturated 10-15x. After sufficient trituration, each well of the plate was washed down with cell lysate and the lysate was transferred to a fresh 1.5ml Eppendorf tube and centrifuged for 1 minute at 10,000xg. After centrifugation the supernatants were carefully transferred to fresh 1.5ml tubes and stored on ice. Next, each sample was quickly vortexed and 100µl of each sample, with the exception of wells treated with 100ng/ml BMP-2 (these wells only received 50µl of sample and 50µl of ALP lysis buffer from section 3.3.2.2), were loaded into to a Costar 96 flat bottom plate (9017). Once the samples were

loaded into the 96-well plate, 100 μ l of SIGMA FAST Reagent was added to each well and the plate was read immediately on a BioTek Power Wave XS microplate reader (model #MQX200R) using the Gen5 data analysis software. Note that because this was an enzymatic assay the enzymatic reaction began immediately following addition of the SIGMA FAST Reagent. Therefore, it was important to read the plate immediately. Measurements were obtained from a kinetic read for 90 minutes, measuring at 10 minute intervals for a total of 10 reads. The absorbance was set at 405 nm. After the ALP activity assay was complete, a protein assay was performed on all samples as per section 3.3.5.2 in order to normalize the data to protein.

3.3.2.4 Fixing cells for Alizarin Red Stain

In order to quantify the calcium content in MPCs using the colorimetric ARS, MPCs were cultured and treated with 0, 10, or 100ng/ml of BMP-2 to induce osteogenesis as described in section 3.3.2.1. After 5 days of differentiation, cells were washed 5x with DPBS then fixed for 1 hour in ice cold 70% ethanol (plate was stored on ice during 1 hour fixing). After 1 hour, the cells were washed an additional 3x with DPBS then the plate was sealed with parafilm and stored at 4°C until staining could be performed.

3.3.2.5 Alizarin Red Stain

ARS was prepared by dissolving 1g of alizarin red power (Alizarin Red S, certified by the Biological Stain Commission – Sigma-Aldrich, St. Louis, MO, cat # A5533-25G) in 50ml of DW. The pH was then adjusted to 4.1 with 6M HCl (hydrogen chloride). Once the ARS was prepared it was filtered using a coffee

filter into a fresh sealable jar and stored at RT in the dark. After preparation of the stain, fixed plates from section 3.3.2.4 were stained as follows: Wells were washed once with 1ml of DW, followed by the addition of 1ml of ARS per well (for a 12-well plate). Plates were then incubated at RT in the dark for 45 minutes. After 45 minutes the stain was aspirated from each well and the cells were washed 4x with 1ml of DW. After washing with DW, 1ml of DPBS was added to each well. At this time, the plates could be observed under the microscope using the 4x objective lens and pictures could be taken. Stained orange regions within the cells indicated presence of calcium deposits. Plates were then stored in the dark at 4°C until ARS quantification could be completed to quantify the calcium deposits within the cells.

3.3.2.6 Alizarin Red Stain Quantification

Once MPCs were stained for ARS, the stain could be quantified. In order to do this, a 10% solution of cetylpyridinium chloride (CPC, lyophilized powder – Sigma-Aldrich, St. Louis, MO, cat # C0732) was prepared as follows: 2g of CPC was added to 20ml of DW (slight heat was needed to help the CPC dissolve). Next, standards for the assay were prepared by making 1ml of a 2mM stock solution of ARS (58.4mM stock ARS was diluted to 2mM with 10% CPC). Standards were prepared by performing serial dilutions of 2mM ARS with 10% CPC for a 1:1 ratio (blank will be 1ml of 10% CPC alone). In order to quantify ARS, plates were removed from the 4°C fridge and the DPBS was aspirated out. Next 1ml of 10% CPC (for a 12-well tissue culture plate) was added to each well and the plate was incubated at RT for 20 minutes. After 20 minutes the solution

in each well was gently mixed by trituration and 200 μ l was loaded, in duplicate, to a 96-well clear bottom plate. The absorbance was read at 550nm on a BioTek Power Wave XS microplate reader (model #MQX200R) using the Gen5 data analysis software.

3.3.3 Experimental overview of specific aim #2

The objective of specific aim #2 was to determine the effects of ascorbic acid on the osteogenesis of MPCs. In order to do this, MPCs were cultured and treated as per section 3.3.2.1 with 0, 10, or 100ng/ml BMP-2 to induce osteogenesis. Then a dose of 100 μ M of ascorbic acid was added to the appropriate wells. BMP-2 treatment media was changed every second day while ascorbic acid was added to the media each day at a dose of 100 μ M. This was because ascorbic acid autoxidizes in the presence of oxygen and is rapidly lost from aqueous solutions at physiological temperature and pH [156]. In order to assess the effects of ascorbic acid on osteogenesis, ALP and ARS quantification assays were conducted as per sections 3.3.2.2 to section 3.3.2.6.

3.3.4. Experimental overview of specific aim #3

The objective of specific aim #3 was to investigate if the actions of ascorbic acid toward MPCs (which were induced to undergo osteogenesis by BMP-2), are due to pro-oxidant activity. In order to accomplish this MPCs were cultured and treated with various doses of antioxidants including TEMPOL or PEG-Cat. After treatment with the appropriate antioxidant, ascorbic acid was added to the wells in order to determine of ascorbic acid functions as a pro-

oxidant. Superoxide production was detected using a Nitroblue Tetrazolium (NBT) assay described in section 3.3.4.1.

After determining if ascorbic acid functions as a pro-oxidant at high doses, MPCs were treated with various doses of BMP-2 as per section 3.3.2.1 as well as the antioxidants described in section 3.3.4.1 and, where appropriate, 0 or 100 μ M ascorbic acid. An ALP assay was then conducted as per sections 3.3.2.2 and 3.3.2.3 in order to determine if the addition of antioxidants TEMPOL and PEG-Cat could reverse the osteogenic inhibition observed by ascorbic acid.

3.3.4.1 Detection of Superoxide Production with the Nitroblue Tetrazolium Assay

In order to determine that ascorbic acid functions as pro-oxidant, cells were seeded at 50k cells in a 12-well tissue culture plate then placed in 6% O₂. Roughly 24 hours after the cells were seeded, the cells were pre-treated with the appropriate antioxidant for 2 hours. Antioxidant treatments included 200U catalase-polyethylene glycol (PEG-catalase, lyophilized powder – Sigma-Aldrich, St. Louis, MO, cat # C4963) or 1mM TEMPOL (4-Hydroxy-TEMPO – Sigma-Aldrich, St. Louis, MO, cat # 176141-1G). After the 2-hour pre-treatment, 1 NBT tablet (Nitro Blue Tetrazolium – Sigma-Aldrich, St. Louis, MO, cat # N5514-25TAB) was dissolved into 1ml of cell culture grade water. This mixture was vortexed until the tablet was fully dissolved. Next, the NBT solution was added to 9ml of warm GM for a final volume of 10ml. At this time, the appropriate concentrations antioxidants, and ascorbic acid (200 μ M) was prepared. A final concentration of 1mM TEMPOL or 200U PEG-Cat was added to 1ml aliquots of

NBT/media mixture for each required well. Then the wells were changed to this treatment media following a DPBS wash. After treatment with the appropriate antioxidant or control media (only NBT+GM), ascorbic acid was added to the wells at a dose of 200 μ M. Plates were then incubated at 37°C in 6% O₂ for 2.5 hours. During this 2.5-hour incubation the plates were observed for colour changes in media indicating superoxide scavenging. After 2.5 hours the plate was washed 2x with DPBS following the addition of 1ml of methanol to each well. The methanol was immediately aspirated out and the wells were allowed to air dry. Next, 240 μ l of 2M KOH was added to each well and the plate was tiled back and forth to ensure complete coverage. Finally, 280 μ l of DMSO was added to each well and the plate was agitated on *The Belly Dancer* at speed 6 for 10 minutes. Following the 10 minute incubation, cell lysates were triturated ~10x and 200 μ l of sample was loaded into to a 96-well clear bottom plate. The plate was read on a BioTek Power Wave XS microplate reader (model #MQX200R) using the Gen5 data analysis software at an absorbance of 620nm.

3.3.5 Experimental overview of specific aim #4

The objective of specific aim #4 was to investigate the effects of ascorbic acid on the myogenic capacity and proliferation of MPCs. For this aim, MPCs were cultured and treated with various doses of ascorbic acid. Myogenesis was assessed using a western blot probing for myosin heavy chain (MHC) protein and a Dual-Luciferase Reporter Assay with muscle creatine kinase (CKM). In order to assess proliferation, BrdU incorporation and flow cytometry was utilized.

3.3.5.1 Cell lysis for myosin heavy chain

Cells were seeded at a density of 50k cells in a 12-well plate and placed in 20% O₂. They were allowed to adhere for 24 hours. Roughly 24 hours later when the cells were ~70% confluent, they were treated with 50µM, 100µM or 150µM of ascorbic acid following a DPBS wash and a media change to fresh GM or DM (2% FBS DMEM HG). Media and treatments were changed each day for 2 days until mature myotubes had formed. Once myotubes formed the cells were lysed for myosin heavy chain as follows: On ice, plates were washed 2x with DPBS. Then 300µl of lysis buffer (300mM KCl pathscan buffer (25 mM Tris (pH 7.5) + 150 mM NaCl + 1 mM EDTA (Ethylenediamine-tetraacetic Acid, Certified ACS – Fisher Scientific, Fair Lawn, NJ, cat # E478-500) + 1% Triton®-X-100) with KCl + 1mM PMSF (Phenylmethanesulfonyl fluoride – Sigma-Aldrich, St. Louis, MO, cat # 78830) was added to each well. Using a cell lysis scraper, each well was thoroughly scraped in all directions to release cells from plastic, then triturated 10-15x. The walls of each well were then washed down with lysis buffer (from the well) and the lysate was transferred to a freshly labelled 1.5ml Eppendorf tube placed on ice. Samples were stored at -80°C until a protein assay could be completed.

3.3.5.2 Protein assay

Before a western blot could be performed, a protein assay was conducted in order to be able to normalize the samples to total protein content. Protein

assays were performed as per the Pierce™ BCA Protein Assay Kit. The samples were first removed from the -80°C freezer and thawed on ice (they were first thawed in a bead bath until a small ice chunk remains, then the remaining thawing took place on ice). Once the samples were completely thawed, they were centrifuged at 4°C at 16,000xg for 10 minutes. Supernatants were then transferred carefully to freshly labelled 1.5ml Eppendorf tubes on ice. Steps hereafter were followed using the Pierce™ BCA Protein Assay Kit described previously: Samples (10µl) were loaded into a Costar 96 flat bottom plate (9017) in duplicate. Once all samples have been loaded 200µl of working reagent (WR: prepared 50:1 for reagent A:B) was added to each well containing a sample. This plate was then mixed on a plate shaker set at 200rpm for 30 seconds. After mixing of the WR and samples, the plate was then incubated at 37°C for 30 minutes. Following the 30-minute incubation, the plate was allowed to cool to RT and the absorbance was read at 562nm on a BioTek Power Wave XS microplate reader (model #MQX200R) using the Gen5 data analysis software.

3.3.5.3 Sample preparation for western blot

Once a protein assay had been performed, the samples were specially prepared for a myosin heavy chain western blot. Firstly, a 1ml aliquot of 4x SDS (Sodium Dodecyl Sulfate, electrophoresis grade – Fisher Scientific, Fair Lawn, NJ, cat # BP166-500) was thawed at RT. Once the 4x SDS was thawed, 110 µl of BME (2-Mercaptoethanol, electrophoresis grade – Fisher Scientific, Fair Lawn, NJ, cat # BP176-100) was added and the reagent was diluted from 4x SDS to 1x

SDS with distilled water (DW). Once the samples had completely thawed (they were stored on ice during entire preparation), 20 μ l of the original protein sample was added to 180 μ l of 1x SDS + BME in a fresh 1.5ml Eppendorf tube and mixed. Using a needle, holes were carefully poked in the top of each of these tubes and samples were boiled at 100°C for 5 minutes. Tubes were then allowed to cool to RT and, following a quick zip-spin, the samples were frozen and stored at -80°C until a western blot could be performed. The original samples (not prepared with 1x SDS + BME) were also frozen at -80°.

3.3.5.4 Western blot (see Appendix 5.1 for full Lees Lab western blot SOP)

See Appendix 1 for full SOP regarding western blot protocol. Gels were prepared at a 5% concentration in order to isolate myosin heavy chain protein. Appropriate samples (as described in section 3.3.5.3) were thawed on ice and 20 μ l of each sample was loaded into each well with the exception of the first well in which 5 μ l of a molecular ladder (Precision Plus Protein™ Dual Colour Standards, Biorad, USA, cat #161-0374) was loaded instead. All gels were run for 1 hour at 100 volts and the blocking solution consisted of a 5% powdered milk (instant skim milk powder purchased from Safeway) in 1XTBST (TBST: Tris Crystallized Free Base, Molecular Biology Grade – Fisher Scientific, Fair Lawn, NJ, cat # BP152-1 + Tris Hydrochloride, BioUltra Pure Grade – Bioshop, Burlington, ON, cat # TRS002.500 + Tween 20 – Bio Rad, Hercules, CA, USA, cat # 170-6531). Each gel was transferred onto an Amersham™Hybond ECL – 0.45 μ m Nitrocellulose Blotting Membrane for nucleic acid and protein application

(GE Healthcare Life Sciences, Germany, cat # RPN303D). All primary and secondary antibodies were prepared in a 5% milk solution. The primary antibody (MF 20, DSHB – AB_2147781 deposited to the DSHB (Developmental Studies Hybridoma Bank, Iowa City, Iowa) by Fischman, D.A) was used at a ratio of 1:1000, while the secondary antibody (Goat anti-mouse IgG, Peroxidase Conjugated from Thermo Scientific, Rockford, IL, USA, cat # 32430) was used at a ratio of 1:100. After completion of the western blot, all gels were stored in roughly 10-20ml of 1xTBST until they could be discarded or stripped and re-probed. Note that steps involving a Ponceau stain were not necessary when doing a myosin heavy chain western blot as only ~1-2 μ g was needed to run the gel which is not enough protein for the stain to adhere to. Blots were analyzed using a Bio-Rad Chemidoc (Bio-Rad Laboratories, Segrate, Italy) and band density was quantified using the Quantity One software program. All density readings were obtained in units of intensity/mm².

3.3.5.5 Plasmids and plasmid preparation

In order to further assess myogenic differentiation of MPCs, a Dual-Luciferase Reporter assay was performed following muscle creatine kinase promoter/reporter transfections. In order to do this the plasmids first needed to be obtained and prepared. The rat muscle creatine kinase (CK-M) promoter construct was cloned by Dr. Lees [157] from genomic DNA isolated from Fischer-344 x Brown Norway F₁ hybrid skeletal muscle by PCR. The cloned promoter was then ligated into the pGL3-Basic firefly luciferase reporter vector

(Promega, Madison, WI). The pRL-null *Renilla* luciferase reporter vector (pRL-CMV) was purchased from Promega.

Plasmids were purified by centrifugation according to the PureYield™ Plasmid Midiprep System manual, section 5.A: DNA Purification by Centrifugation. Bacterial cultures were started from a glycerol stock consisting of *E. coli* (DH5α strain) cells transformed with the appropriate reporter constructs. Cultures were grown in flame-sterile tubes containing 3ml of LB broth (Molecular Genetics Powder – Fisher Scientific, Fair Lawn, NJ, cat # BP1427-2) for 6 hours at 37°C, shaken at 150 rpm. During the 6 hours, 100ml of LB broth + 100µg/ml ampicillin was aliquoted into an autoclaved 250ml flask and warmed at 37°C. After 6 hours, 1 ml of each starter culture was added to the 250ml flask and placed overnight on a bacterial shaker set at 150 rpm and 37°C. The remainder of the procedure was then followed according to the PureYield™ Plasmid Midiprep System manual, section 5.A: DNA Purification by Centrifugation. This system resulted in roughly 1ml of pure plasmid. In order to determine the concentration of the plasmid a Take3 plate and the A260:A280 method was used. Both sides of the Take3 plate were cleaned with 70% ethanol and 1 µl of nuclease-free water (blank) and 1µl of plasmid sample were loaded then read using a BioTek Power Wave XS microplate reader (model #MQX200R). Measurements were obtained using the Gen5 data analysis software.

3.3.5.6 Transfection with muscle creatine kinase promoter/reporter plasmid into cells

Once the plasmids were prepared (as per section 3.3.5.5), they had to be transfected into the cells. MPCs were seeded in a 12-well tissue culture plate at a density of 50k cells in 20% O₂. Once the cells reached ~70% confluency, they were transfected using a 10mM PEI (Poly(ethyleneimine) solution, Sigma-Aldrich, St. Louis, MO, cat #P3143) transfection reagent. The plasmids used for transfection were prepared a day or two ahead of time and stored at 4°C. The muscle creatine kinase plasmid (CKM-luc) was used at 0.95µg and the *Renilla* plasmid (pRL-CMV) was used at 0.05µg for a total of 1µg of plasmid DNA. The volumes of each plasmid needed were calculated based on the total number of wells. See Appendix 6.3.3.3 for example calculations. In order to transfect the plasmids into the cells, the following procedure was used: Firstly, the appropriate volumes of 10mM PEI and 150mM NaCl (calculations in Appendix 6.3.3.3) were added to a 1.5ml Eppendorf tube labelled Tube#2. Once prepared, the entire volume of Tube#2 (containing PEI transfection reagent) was added to Tube#1 (containing plasmids and appropriate 150mM NaCl volume) and immediately vortexed (important not to add in the reverse order). The transfection solution was then allowed to sit for 10 minutes at RT. During this 10-minute incubation, the wells were washed with DPBS and 1ml of fresh pre-warmed DM was added. After allowing the transfection solution to sit for 10 minutes, 100 µl of reagent was added to each well containing fresh DM and plates were mixed by swirling gently. Next, plates were incubated at 37°C for 2 hours. After the 2-hour incubation,

plates were washed with DPBS and the media was changed to warm DM or GM. At this time, each well was then treated with 50 μ M, 100 μ M or 150 μ M of ascorbic acid and incubated at 37°C. Cells were allowed to differentiate for 2 days until mature myotubes formed. The media and ascorbic acid treatments were changed each day up until that point.

3.3.5.7 Lysing cells for dual-luciferase assay

Following 2 days of differentiation, or formation of mature myotubes, the cells were lysed using 1x PLB (Passive Lysis Buffer, 5X – Promega, Madison, WI, USA, cat # E194A). This PLB was prepared ahead of time from a 5x PLB stock and stored at 4°C (stable at this temperature for weeks). In order to obtain a cell lysate, the plates were washed once with DPBS, then 250 μ l of 1x PLB was added. Following addition of lysis buffer, the plates were placed on *The Belly Dancer* set at speed 6 for 15 minutes. After 15 minutes, the plates were sealed with parafilm and frozen immediately at -80°C until a Dual-Luciferase Reporter assay could be performed.

3.3.5.8 Dual-Luciferase Reporter assay as per Luciferase Assay SOP (Appendix 2)

Firefly and *Renilla* luminescence was measured using the Dual-Luciferase Reporter Assay System (Promega) as per the Luciferase Assay SOP (refer to Appendix 2). All reagents that were used to complete the assay such as, LAR II, Stop & Glo buffer, and Stop & Glo substrate were obtained from the Dual-

Luciferase® Reporter 1000 Assay System purchased from Promega, Madison, WI, cat # E1980.

3.3.5.9 Pulse and fixing for BrdU incorporation

In order to assess if ascorbic acid had an effect on proliferation of MPCs BrdU (bromodeoxyuridine) incorporation and flow cytometry was utilized. Cells were first seeded at a low density (50k cells) in 60mm tissue culture dishes and placed in 6% O₂. Once cells reached roughly 40% confluency (approximately 2 days after seed), the media in each well was changed to fresh GM. At this time, cells in separate 60mm tissue culture dishes were treated with 0, 50µM, 100µM, 200µM, or 500µM ascorbic acid (where the 0µM dose functioned as a control plate). Exactly 24 hours after treatments were applied, the cells were pulsed and fixed with BrdU as follows: 10mM BrdU (5-Bromo-2'-deoxyuridine, Sigma Aldrich, St. Louis MO, USA, cat# B9285) was prepared by diluting the BrdU 1:10 and mixing. BrdU was then added to the media in each culture dish (media did not need to be changed here) at a ratio of 1:100 and the dishes were pulsed for 30 minutes at 37°C in 6% O₂. After 30 minutes the BrdU solution was removed and the 60mm dishes were washed with DPBS. Next the appropriate amount of trypsin-based cell dissociation enzyme solution was added (0.5ml for 60mm dish) to remove the cells from the dishes. Once the cells had detached from the plastic, the trypsin-based cell dissociation enzyme solution was neutralized with 1ml of pre-warmed GM (2x the volume of enzyme solution). The trypsin-based cell dissociation enzyme solution and neutralizing media was then fully collected

into 15ml conical tubes and centrifuged at 150xg for 7 minutes. Following this centrifugation, the supernatants were carefully poured off and the cells were re-suspended in 200µl of DPBS. Once the cells were completely re-suspended they were fixed using an ethanol-based method. Drop-wise, whilst vortexing (speed 5), 4ml of ice cold 70% ethanol was added to each 15ml tube. These tubes were then stored at 4°C for at least for 24 hours until part 2 (flow cytometry) could be completed. The remainder of the BrdU protocol can be found as an SOP in Appendix 6.5. Flow cytometry was run as described in section 3.3.1.4.

3.3.6 Experimental overview of specific aim #5

The final aim of this project was to determine if there was a difference in osteogenic capacity between MPCs and a sub-population of cells where the highly myogenic cells have been removed. In order to investigate this the subpopulation of cells first had to be isolated. This was accomplished using the procedure described below in section 3.3.6.1. Following isolation of the subpopulation, cells were either fixed for pax7 experiments as per section 3.3.1.1 or re-seeded into a 12-well plate at 50k cells per well. They were then treated with 0 or 10ng/ml BMP-2 as per section 3.3.2.1 and an ALP activity assay was performed after 3 days of osteogenic differentiation.

3.3.6.1 Isolation of a subpopulation of MPCs to remove highly myogenic cells

MPCs were seeded at a density of 250k cells in two T25 flasks. Once cells had reached ~70% confluency they were induced to undergo myogenic differentiation with DM. After approximately 2 days of differentiation, or after

sufficient myotubes had developed, the cells were removed from the flasks using a trypsin-based cell dissociation enzyme solution as in section 3.3.1.1. After neutralizing cells with DM, the cells + media + trypsin-based solution was collected into a serological pipette and strained through a 100 μ m nylon mesh sterile cell strainer to remove the myotubes. The remaining myoblasts which passed through the membrane were then centrifuged at 150xg for 7 minutes. The pellet was then fixed for pax7 experiments (see section 3.3.1.1 for fixing) or re-suspended in 1ml of GM and counted. The counted cells were then used to re-seed 12-well plates at 50k cells for BMP-2 treatments and ALP assays in order to further investigate this specific isolated population of MPCs.

3.4 Statistical Analysis

All data are presented as means \pm SEM. All statistical analyses were performed using GraphPad Software (Prism 7.01 version, GraphPad Software, Inc. La Jolla, CA, USA. 2016). A resulting p-value of less than or equal to 0.05 was considered to be significant. Data sets were analyzed using one-way ANOVA followed by a Fishers LSD post hoc. Additional statistical analysis was performed (data not shown) using one-way ANOVA with repeated measures followed by a Tukey post hoc analysis.

Chapter 4. Results

4.1 Assessing the purity of MPCs

Pax7 is a paired box transcription factor that is expressed specifically in quiescent and proliferating satellite cells. As such it is frequently used in the

literature as a marker of satellite cells [158, p. 7]. Pax7 was therefore chosen to assess the purity of MPCs following isolation. While the methods utilized in this study (refer to section 3.1.1) to isolate MPCs have been well established in literature, it was important to verify the purity of primary cell isolation for these experiments [159], [160].

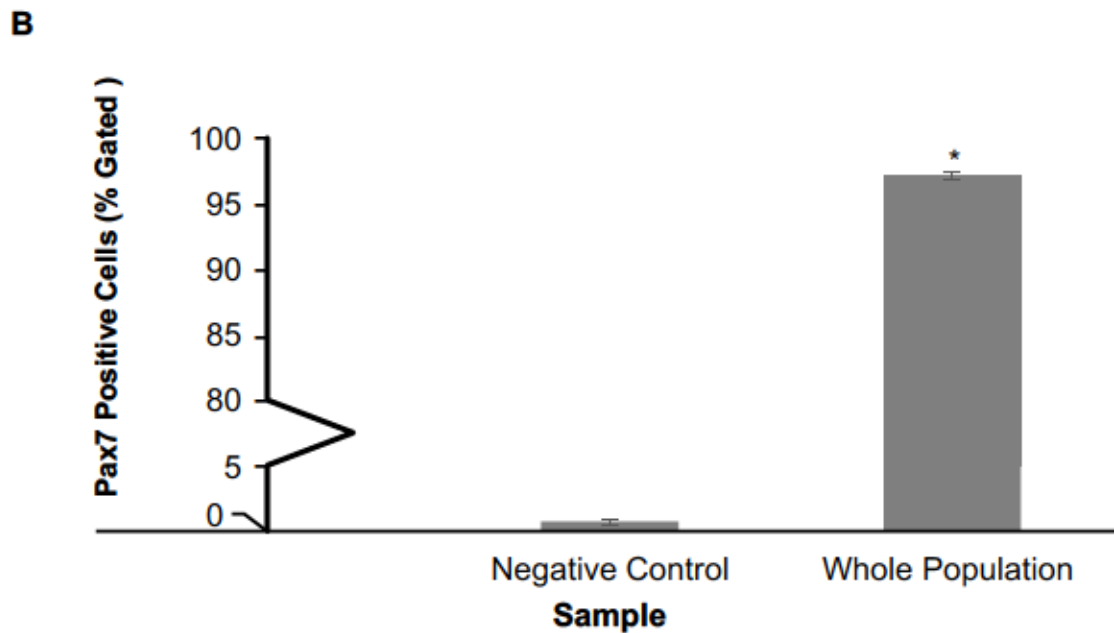
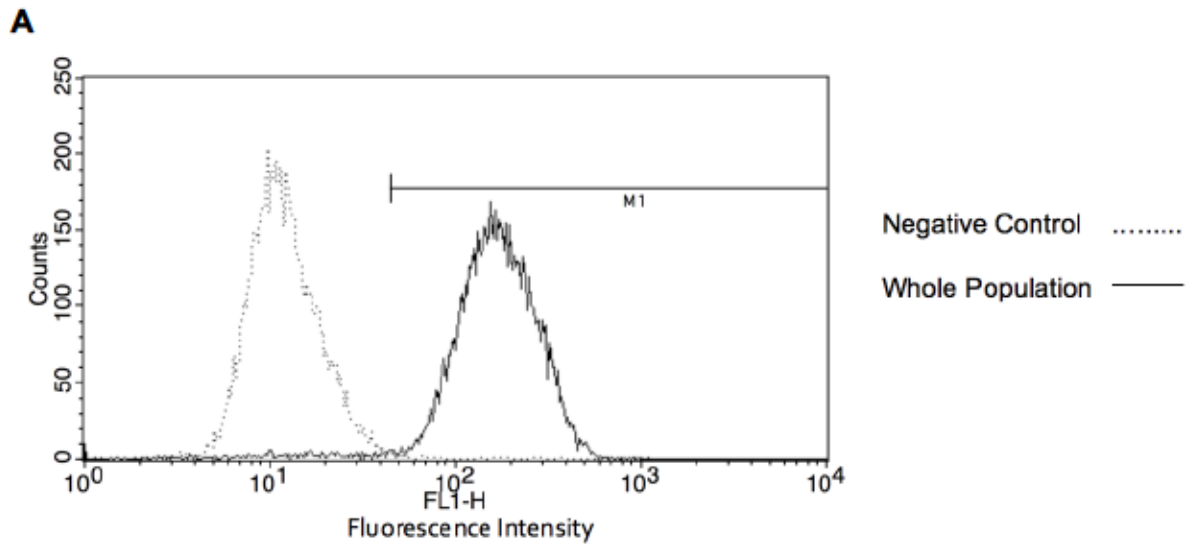


Figure 10. **Purity assessment of MPCs using Pax7 immunofluorescence.** In order to determine if the cells isolated for this study were MPCs (muscle precursor cells) the satellite cell marker Pax7 was utilized followed by flow cytometry analysis. As viewed in Figure 10.A, a histogram was constructed of MPCs displaying two independent peaks. The dotted line represents the negative control (which were not subject to Pax7 immunofluorescence) while the solid line represents the normal or “whole population” of cells (subject to Pax7 immunofluorescence). The M1 population represents the Pax7⁺ population of MPCs based on the overall percentage of Pax7⁺ cells. In Figure 10.B, group mean data for the percentage of MPCs positive for Pax7 within the defined gated region are displayed. In the negative control, 0.74% of cells were Pax7⁺, while the whole population of cells were approximately 98.09% Pax7⁺ (n=4). A break in the axis is denoted by >, and significance difference from control is denoted by *, (p≤0.05).

4.2 Experimental results of specific aim #1

The objective of specific aim #1 was to investigate the potential of MPCs to be induced into a bone lineage. This was accomplished by treating the cells with either 0, 10 or 100ng/ml of BMP-2 (see section 3.3.2) then assessing osteogenesis using an ALP assay as well as an ARS quantification method.

ALP is an ectoenzyme that is anchored to the cell surface of osteoblasts as well as other cell types. It is frequently used in the literature as a marker for bone formation and to monitor bone metabolism [161]. As such, this marker was used to assess if MPCs were capable of being induced to an osteoblastic cell lineage. Figure 11 illustrates the differences in ALP activity of control cells and cells treated with 100ng/ml of BMP-2. As viewed below, there was an ~2-fold increase in ALP activity between control and BMP-2 treatment.

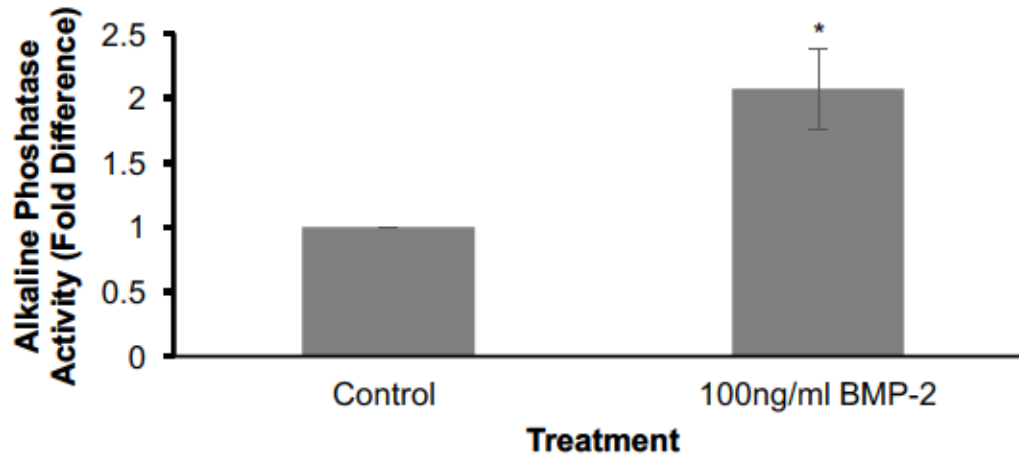


Figure 11. **Alkaline phosphatase activity of MPCs treated with BMP-2.** In order to determine if MPCs (muscle precursor cells) were capable of undergoing osteogenic differentiation they were treated with 100ng/ml of BMP-2 (bone-morphogenetic protein-2) and ALP (alkaline phosphatase) activity was assessed. Data were expressed in terms of fold difference of MEAN \pm SEM (n=6). Statistical significance from control is denoted by *, ($p \leq 0.05$).

Colorimetric quantification of calcium deposits was assessed using alizarin red dye as viewed in Figure 12. MPCs were treated with 10ng/ml BMP-2 every 48 hours and allowed to differentiate for a total of 5 days. On the fifth day the cells were fixed and stained for ARS. Following staining, the amount of stain bound to calcium was quantified using 10% CPC (see section 3.3.2). CPC will penetrate the cell membrane, releasing the contents within the cell. As such this chemical was used to quantify how much ARS was bound to calcium and assess osteogenesis. As viewed below there was no significant increase in calcium content between control and the BMP-2 treatment.

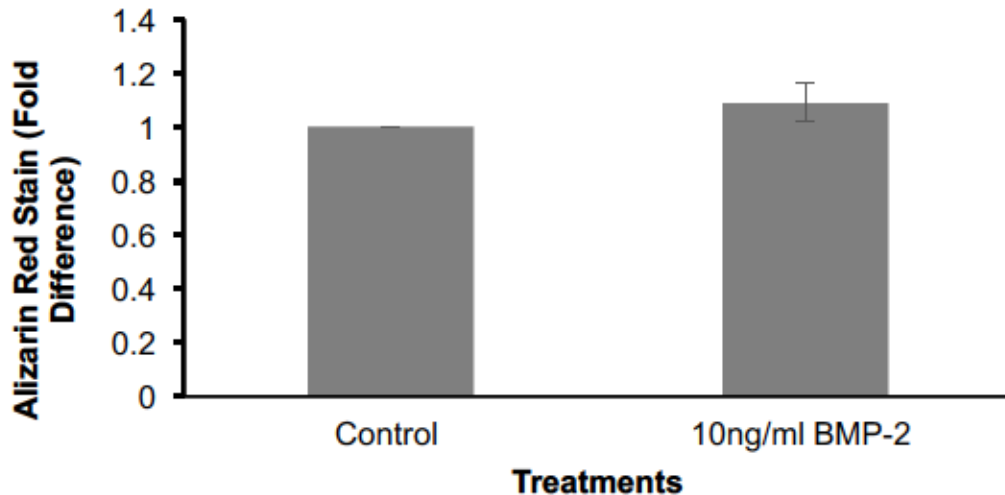


Figure 12. **Alizarin red stain quantification of MPCs treated with BMP-2.** In order to further assess the osteogenic ability of MPCs (muscle precursor cells), cells were first treated with 10ng/ml of BMP-2 (bone morphogenetic protein-2) and then after 5 days stained for presence of calcium deposits. The amount of calcium present in each sample was then quantified using an alizarin red stain (ARS) quantification method. Data are expressed in terms of fold difference of MEAN \pm SEM (n=6). It was found that there was no statistical difference between control and 10ng/ml BMP-2 treated cells.

4.3 Experimental results of specific aim #2

The objective of specific aim #2 was to determine the effects of ascorbic acid on the osteogenic capacity of MPCs. As described in section 3.3.3, MPCs were treated with either 0, 10 or 100ng/ml of BMP-2 then treated with 0 or 100 μ M ascorbic acid. Osteogenesis was then assessed using an ALP activity assay as well as an ARS quantification method. This approach was taken in order to determine the effects of a high dose of ascorbic acid (100 μ M) on MPCs. When ALP activity was assessed, it was found that (as described in section 4.2) 100ng/ml BMP-2 resulted in a statistically significant increase (~2-fold) increase

in ALP activity. When MPCs were treated with 100 μ M ascorbic acid in combination with 100ng/ml BMP-2, no increase in ALP activity was observed (Figure 13). These data indicate that ascorbic acid blocks the osteogenic effect of BMP-2 on MPCs. Upon treatment of cells with 10ng/ml BMP-2 and 100 μ M ascorbic acid, there was no statistical difference found between treatments when ARS quantification was performed (Figure 14).

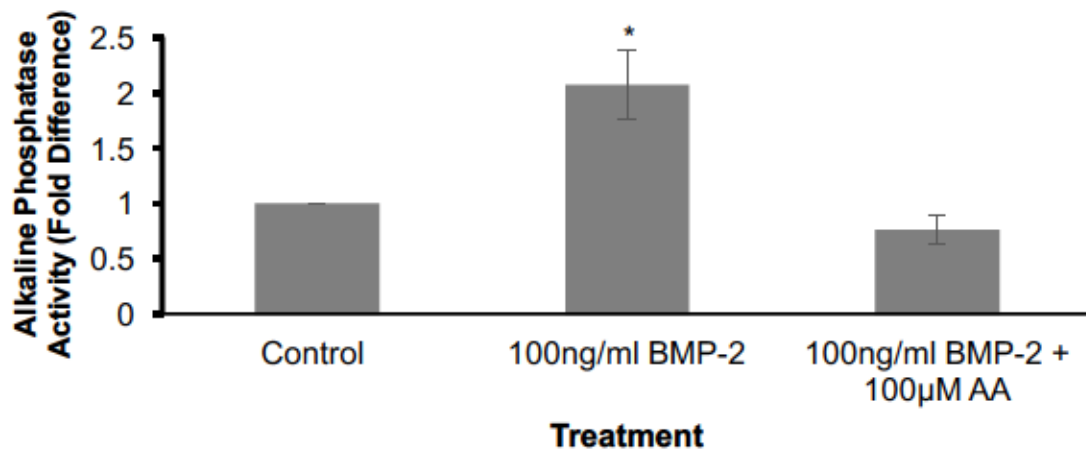


Figure 13. **Determining effects of ascorbic acid on MPCs through ALP activity.** In order to determine if ascorbic acid (AA) would affect BMP-2 (bone morphogenetic protein-2)-induced osteogenesis in MPCs (muscle precursor cells), cells were treated with 100ng/ml BMP-2 or 100ng/ml BMP-2 + 100 μ M ascorbic acid. When treated with 100ng/ml BMP-2, ALP (alkaline phosphatase) activity significantly increased ($p \leq 0.05$). When a dose of 100 μ M ascorbic acid was then added in combination with the BMP-2 treatment, there was no significant increase in ALP activity. This suggests that ascorbic acid blocks the osteogenic effect of BMP-2 on MPCs. Statistical significance from control is denoted by *, ($p \leq 0.05$). Data is expressed in terms of fold difference of MEAN \pm SEM (n=6).

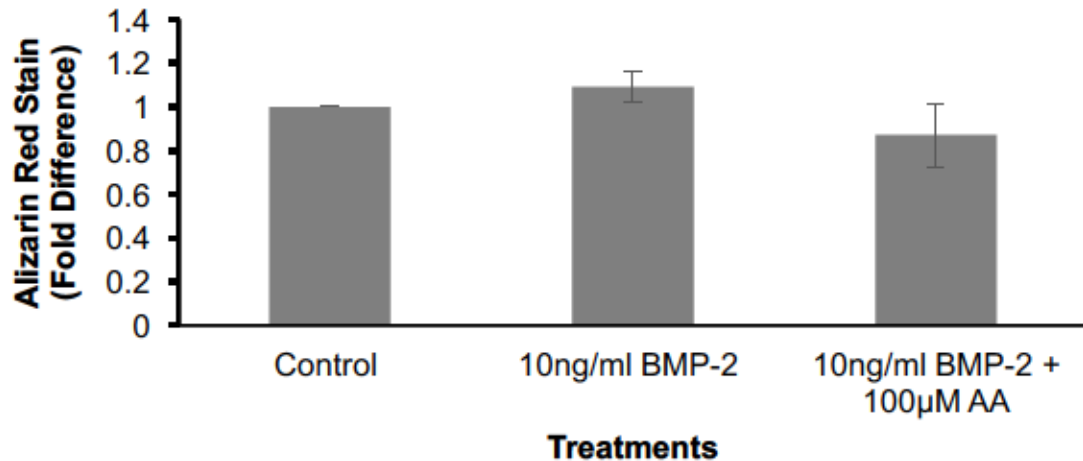


Figure 14. **Determining effects of ascorbic acid on MPCs using ARS quantification.** In order to determine the effects of ascorbic acid (AA) on MPCs (muscle precursor cells), cells were treated with 10ng/ml BMP-2 (bone morphogenetic protein-2) or 10ng/ml BMP-2 + 100µM ascorbic acid. Cells were then allowed to differentiate for 5 days followed by ARS (alizarin red stain) and quantification. Data are expressed in terms of fold difference of mean \pm SEM (n=6). There was no statistical difference found between any of the treatments given.

4.4 Experimental results of specific aim #3

4.4.1 Determining if ascorbic acid functions as a pro-oxidant

The objective of specific aim #3 was to investigate if the actions of ascorbic acid on MPCs, which have been induced to undergo osteogenesis by BMP-2, are due to pro-oxidant activity. The first step was to determine if ascorbic acid acted as a pro-oxidant at high doses. This was investigated using an NBT assay which measures the production of superoxide (a type of ROS). MPCs were treated as per section 3.3.4 with two different antioxidants (1mM Tempol or 200U PEG-Cat) and/or 200µM ascorbic acid. The treatment dose of ascorbic acid for this particular assay was established from preliminary unpublished laboratory

data as described in Appendix 6.7. As viewed in Figure 15, when MPCs were treated with a dose of ascorbic acid (200 μ M), superoxide production was increased to a statistically significant difference ($p \leq 0.05$). When the cells were pretreated with 1mM TEMPOL in addition to ascorbic acid, there was no increase in superoxide production. There was no statistical difference between control and 1mM TEMPOL + 200 μ M ascorbic acid treatments however there was a statistically significant decrease between 200 μ M ascorbic acid (which functioned as the positive control) and 1mM TEMPOL + 200 μ M ascorbic acid ($p \leq 0.05$). Because TEMPOL functions as an SOD mimic, meaning that it scavenges superoxide, these data indicate that there may be superoxide production by ascorbic acid. Conversely, when the antioxidant PEG-Cat was introduced at a dose of 200U in combination with the 200 μ M ascorbic acid treatment, superoxide production increased. There was a statistical difference observed between control and 200U PEG-Cat + 200 μ M ascorbic acid treatment ($p \leq 0.05$). There was no statistical difference between 200 μ M ascorbic acid and 200U PEG-Cat + 200 μ M ascorbic acid treatment. There was a statistical difference found between the two antioxidant treatments however: 1mM TEMPOL + 200 μ M ascorbic acid versus 200U PEG-Cat + 200 μ M ascorbic acid ($p \leq 0.05$).

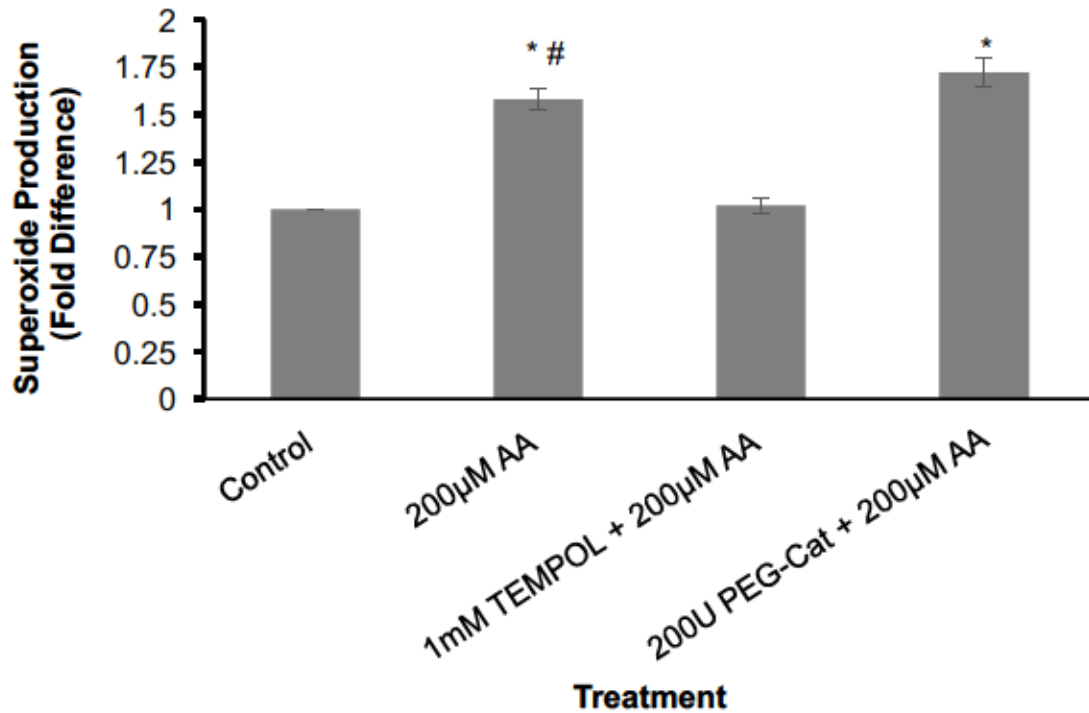


Figure 15. **Assessment of pro-oxidant potential of ascorbic acid.** In order to determine if ascorbic acid (AA) functions as a pro-oxidant at elevated doses, cells were treated with a 200µM dose of ascorbic acid. This then served as a positive control when cells were further treated with the antioxidants TEMPOL (4-Hydroxy-TEMPO) or PEG-Cat (polyethylene glycol-catalase). Pro-oxidant potential was assessed using an NBT (Nitroblue Tetrazolium) assay which measures superoxide production. Data are expressed in terms of fold difference of mean \pm SEM (n=6). Significant differences from control are denoted by *, and significant differences from 1mM TEMPOL + 200µM AA are denoted by #, ($p \leq 0.05$).

4.4.2 Determining if ascorbic acid blocks the effects of BMP-2 due to pro-oxidant activity

In order to determine if ascorbic acid blocks the effects of BMP-2 due to pro-oxidant activity, MPCs were treated with the same doses of antioxidants and ascorbic acid as in section 4.4.1 and an osteogenic assay was performed. As described in section 3.3.4, MPCs were induced to undergo osteogenesis with

100ng/ml BMP-2 then treated with either 1mM TEMPOL or 200U PEG-Cat. As viewed in Figure 16 when MPCs were treated with 100ng/ml BMP-2 alone, ALP activity significantly increased by ~2-fold ($p \leq 0.05$). When ascorbic acid (100 μ M) was added in combination with BMP-2 treatment, there was no increase in ALP activity. There was no statistical difference between control versus 100ng/ml BMP-2 + 100 μ M ascorbic acid treatment. Following pretreatment with 1mM TEMPOL to the BMP-2 and ascorbic acid treated cells, ALP activity significantly increased relative to the 100ng/ml BMP-2 + 100 μ M ascorbic acid treatment ($p \leq 0.05$). Additionally, there was no statistical difference found between 100ng/ml BMP-2 treatment and 100ng/ml BMP-2 + 100 μ M ascorbic acid + 1mM TEMPOL treatment. Conversely, when MPCs were treated with 200U PEG-Cat and 100 μ M ascorbic acid, in combination with 100ng/ml BMP-2, ALP activity was not different from control. There was no statistical significant difference found between 100ng/ml BMP-2 + 100 μ M ascorbic acid versus 100ng/ml BMP-2 + 100 μ M ascorbic acid + 200U PEG-Cat.

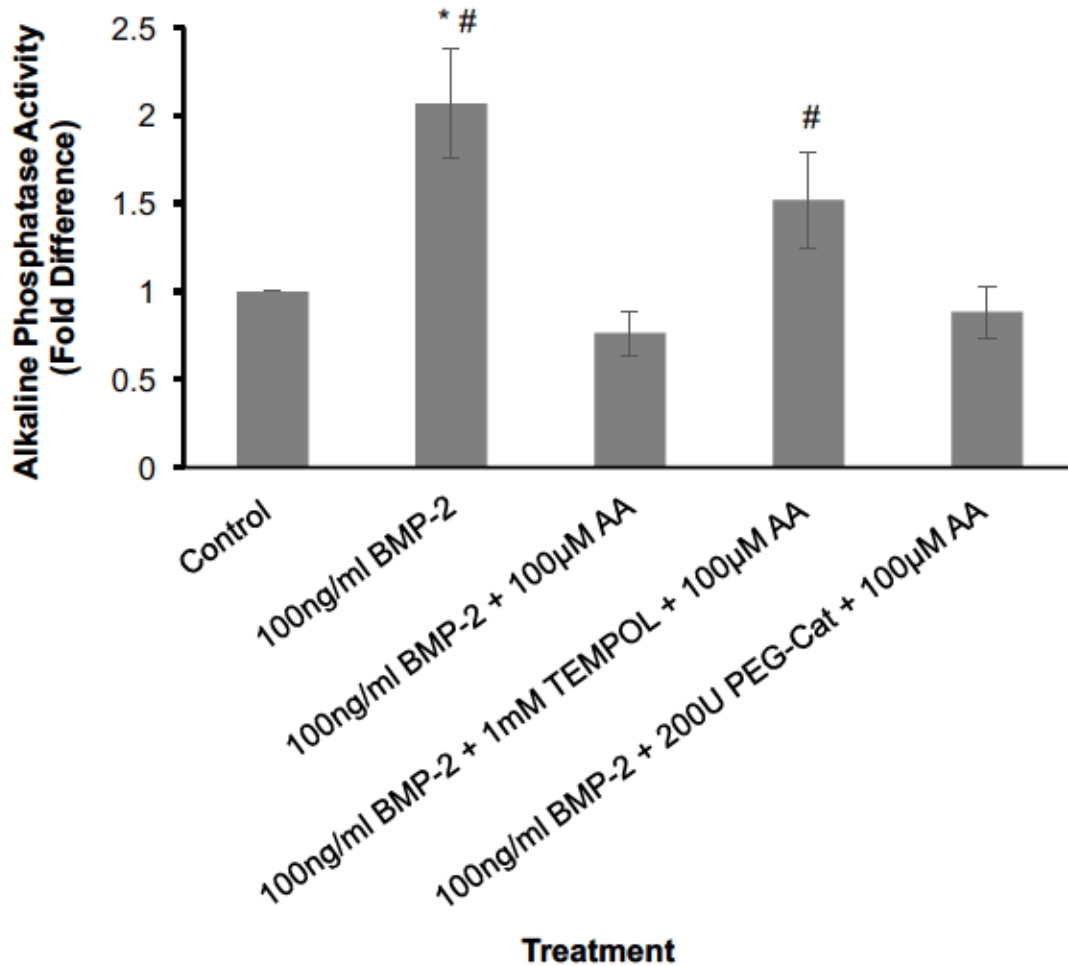


Figure 16. **Pro-oxidant effects of ascorbic acid on MPCs which have been induced to undergo osteogenesis with BMP-2.** In order to assess if the osteogenic reversal observed by ascorbic acid (AA) on MPCs (muscle precursor cells) was due to pro-oxidant activity, cells were induced to undergo osteogenesis with 100ng/ml BMP-2 (bone morphogenetic protein-2), then treated with 100µM ascorbic acid in combination with either 1mM TEMPOL (4-Hydroxy-TEMPO), or 200U PEG-Cat (polyethylene glycol-catalase). ALP (alkaline phosphatase) activity assays were then performed to assess changes in osteogenic potential. Data are expressed in terms of fold difference of mean \pm SEM (n=6). Significant difference from control is denoted by *, while significant differences from 100ng/ml BMP-2 + 100µM ascorbic acid treatment are denoted by #, ($p \leq 0.05$).

4.5 Experimental results of specific aim #4

4.5.1 Effects of ascorbic acid on cell fusion and myotube formation of MPCs

The objective of specific aim #4 was to investigate the effects of ascorbic acid on the myogenic capacity and proliferation of MPCs. Myogenic differentiation was induced two different ways: using a low-serum differentiation medium, or by allowing the cells to reach confluency in a mitogen rich growth medium to initiate the differentiation program.

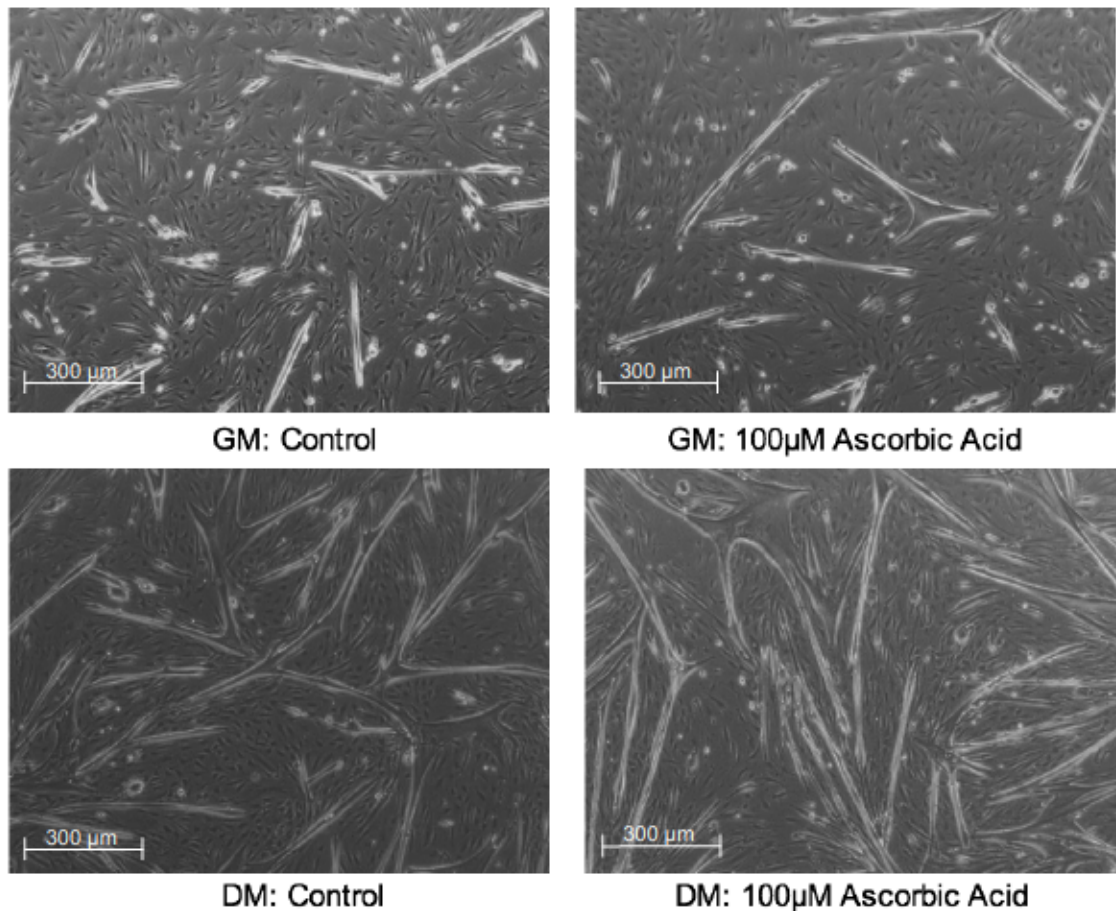


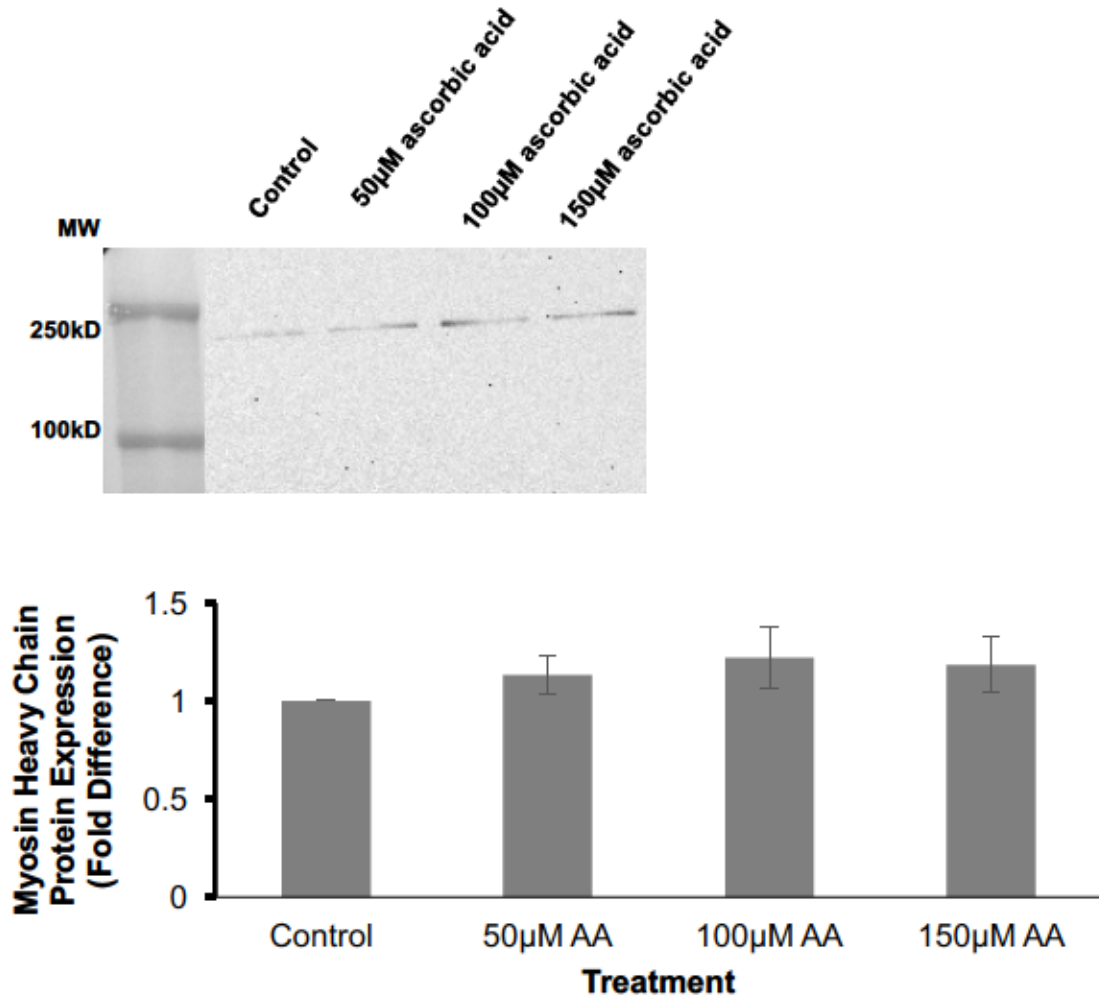
Figure 17. **Myogenic differentiation of MPCs treated with ascorbic acid.** In order to partially assess if high doses of ascorbic acid had an effect on myogenic differentiation of MPCs (muscle precursor cells), images were taken of MPCs which either received no treatment, or were treated with 100 μ M ascorbic acid (AA). Images were taken of cells differentiated for 2 days in either growth

medium (GM) or differentiation medium (DM) using phase contrast microscopy with 4x objective lens magnification. As viewed in the figure, there were no detectable differences between myotube formation between control and ascorbic acid treatments in both GM and DM.

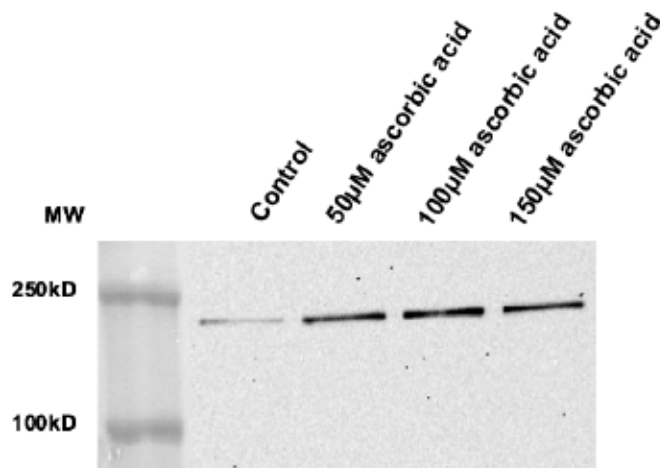
4.5.2 Ascorbic acid effects on myogenesis with myosin heavy chain western blot

In order to further assess the effects of ascorbic acid on myogenic capacity of MPCs, western blots probing for myosin heavy chain were performed on differentiated cells as per section 3.3.5. Myosin heavy chain is the motor protein of muscle thick filaments [162]. Figure 18 illustrates the effects of various doses of ascorbic acid on myogenesis of MPCs after differentiation using either GM or DM. The use of GM forces differentiation based on confluency, while DM forces cells to enter a differentiation program with a low-serum concentration. Western blots were performed and band density was quantified in terms of intensity/mm² in order to assess the effects of ascorbic acid on myosin heavy chain protein. There were no statistically significant differences between control and any of the doses of ascorbic acid (50µM, 100µM or 150µM) when cells were differentiated using GM or DM. There was however a statistically significant difference found between treatments when administered in GM versus DM ($p \leq 0.05$).

A



B



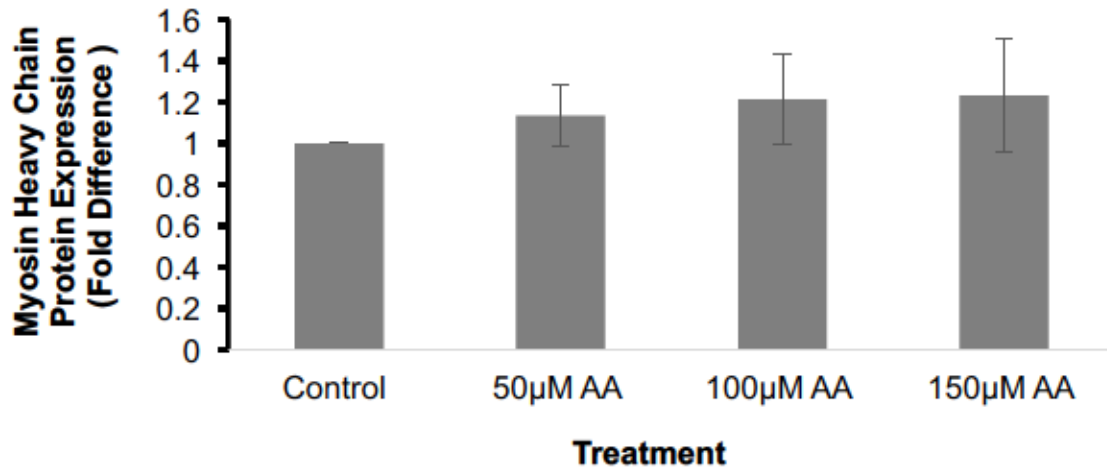


Figure 18. **Myosin heavy chain western blot to examine the effects of ascorbic acid on myogenic differentiation of MPCs.** In order to assess if ascorbic acid (AA) had an effect on myogenic differentiation of MPCs (muscle precursor cells), cells were treated with either 0, 50µM, 100µM or 150µM of ascorbic acid. They were allowed to differentiate either in growth media (GM) (Figure 18.A), by reaching confluency, or in differentiation medium (DM) (Figure 18.B) for 2 days or until mature myotubes formed. Western blots were then performed and band density was quantified in terms of intensity/mm² indicating myosin heavy chain protein. Data are expressed in terms of fold difference of mean \pm SEM (n=6). As viewed in Figure 18.A, there were no statistically significant difference between control and ascorbic acid treatments, indicating no effect on differentiation. Similar results were observed in Figure 18.B when cells were differentiated in DM. A representative myosin heavy chain (MHC) western blot is included as well as group mean data for cells cultured in both GM and DM. There were no statistically significant differences found between control and ascorbic acid treatments, indicating no effect on differentiation.

4.5.3 Ascorbic acid effects on muscle creatine kinase expression

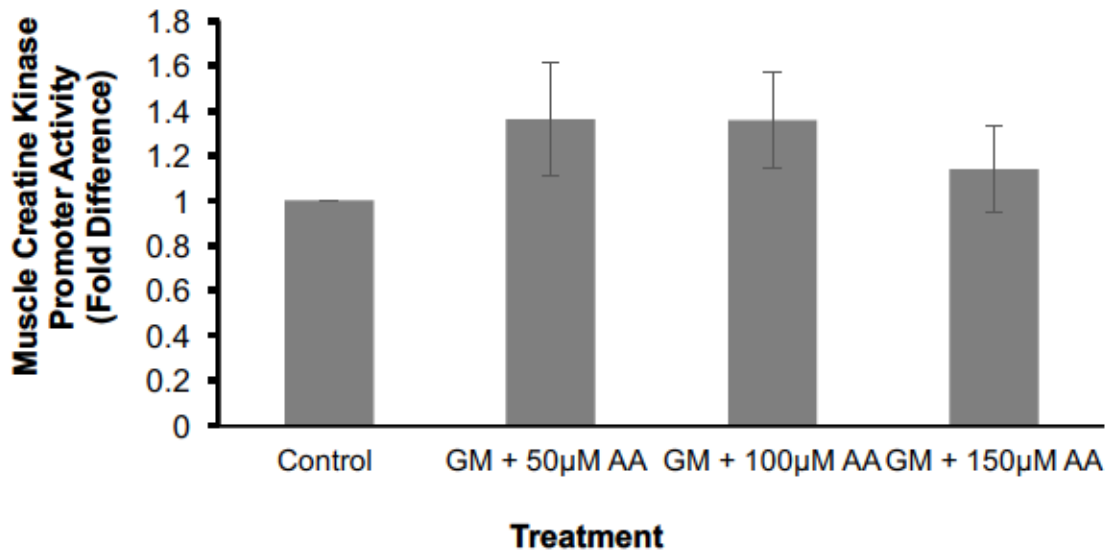
Myogenesis was additionally assessed via transfections with a muscle creatine kinase promoter and the dual-luciferase reporter assay as per section

3.3.5. Muscle creatine kinase is a protein expressed in muscle. Figure 19

illustrates the effects of ascorbic acid on myogenesis of MPCs at doses of 50µM, 100µM and 150µM when cells were differentiated using GM and high confluency

or in DM. Cells were transfected with muscle creatine kinase and allowed to differentiate for 2 days, or until mature myotubes were observed.

A



B

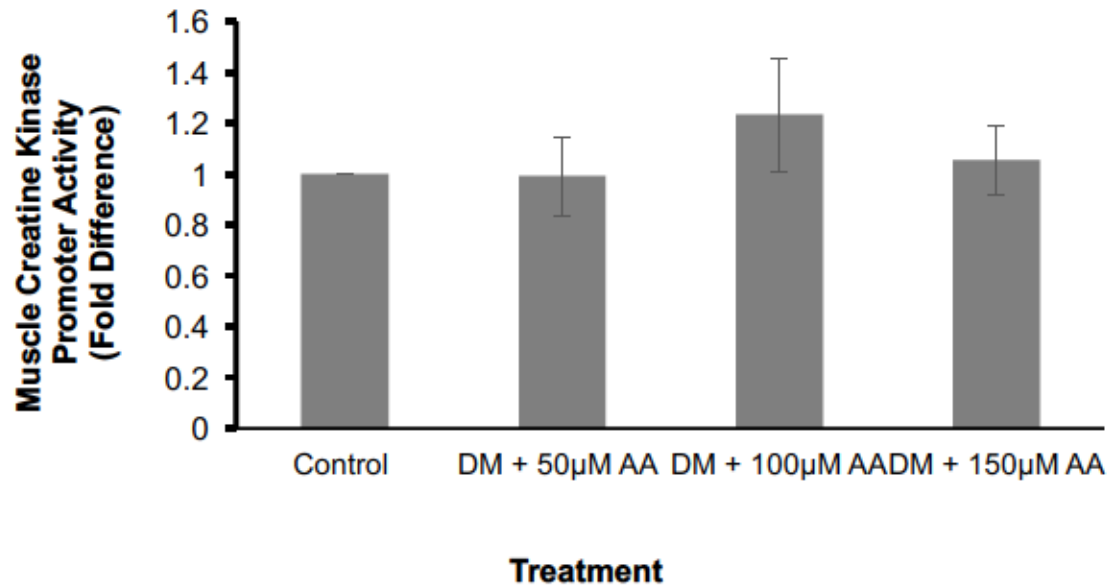


Figure 19. **Dual-luciferase reporter assay with muscle creatine kinase to assess the effects of ascorbic acid on myogenic differentiation.** As a final measurement of the effects of ascorbic acid (AA) on differentiation of MPCs

(muscle precursor cells), cells were transfected with a muscle creatine kinase promoter and, after 2-days of differentiation, a dual-luciferase reporter assay was performed. Data are expressed in terms of fold difference of relative Firefly/Renilla luminescence mean \pm SEM (n=6). As viewed in Figure 19.A, when cells were differentiated in growth media (GM), there were no statistically significant differences observed between control and any of the ascorbic acid treatments (50 μ M, 100 μ M or 150 μ M). The same results were observed in Figure 19.B when cells were differentiated in differentiation medium (DM). No statistically significant differences were observed between control and any of the ascorbic acid treatments, indicating no effect on myogenic differentiation.

4.5.4 Ascorbic acid effects on proliferation using BrdU incorporation

In order to assess the effects of ascorbic acid on proliferation of MPCs BrdU incorporation was utilized as per section 3.3.5. Cells were treated with a variety of high doses of ascorbic acid including 0, 50 μ M, 100 μ M, 200 μ M and 500 μ M. Figure 20.A illustrates a general overlay of untreated control MPCs and 100 μ M ascorbic acid treated cells. Figure 20.B illustrates group mean data, in terms of fold difference, of the amount of BrdU positive cells when treated with various doses of ascorbic acid. All doses of ascorbic acid resulted in no statistically significant difference between treatment and control with the exception of 500 μ M ascorbic acid ($p \leq 0.05$). It should be noted however that at this dose there was significant overall cell loss (>50%).

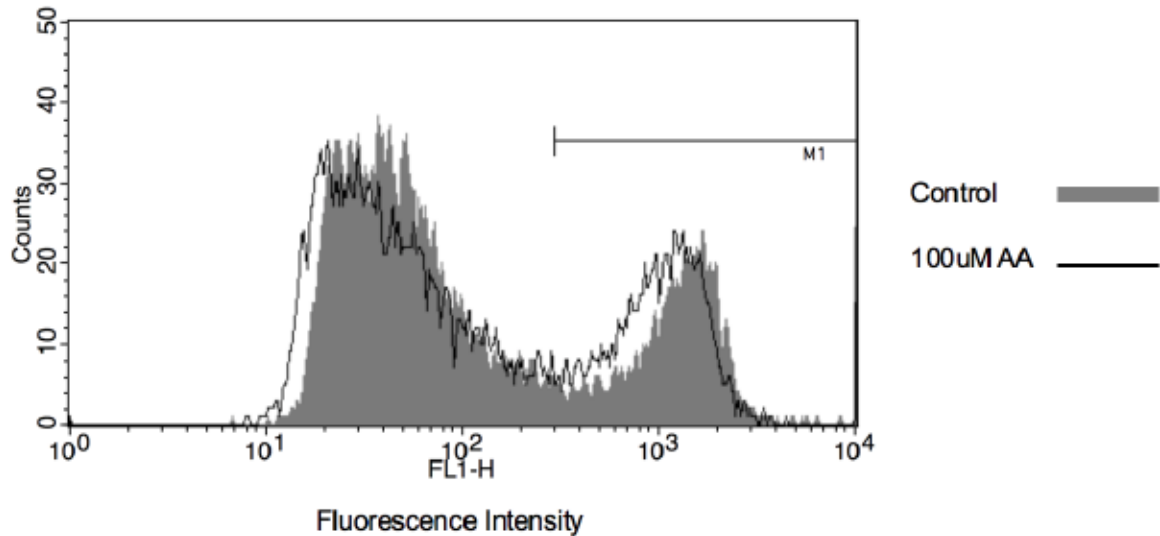
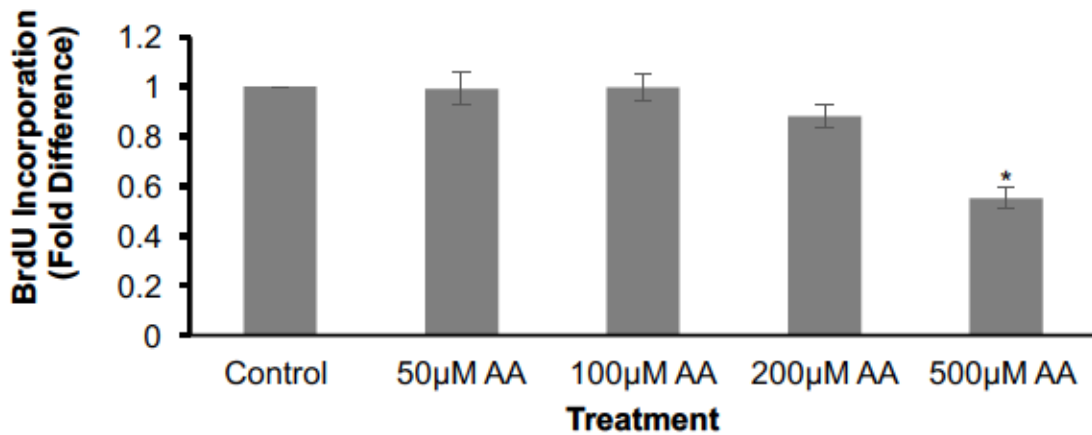
A**B**

Figure 20. **Effects of ascorbic acid on proliferation of MPCs utilizing BrdU incorporation.** Figure 20.A depicts a general overlay constructed following treatment of MPCs (muscle precursor cells) with 0 or 100µM ascorbic acid (AA) and flow cytometry analysis. The grey region is representative of the control sample of MPCs which received no treatment, while the region outlined in a solid black line depicts MPCs that were treated with 100µM ascorbic acid. The M1 line represents the population of cells that are BrdU positive. Figure 20.B illustrates group mean data of the dose response of ascorbic acid treatments represented by fold difference of mean \pm SEM (n=6) of the percentage of BrdU (bromodeoxyuridine) positive cells within the defined gated region. Significant difference from control is denoted by *, ($p \leq 0.05$).

4.6 Experimental results of specific aim #5

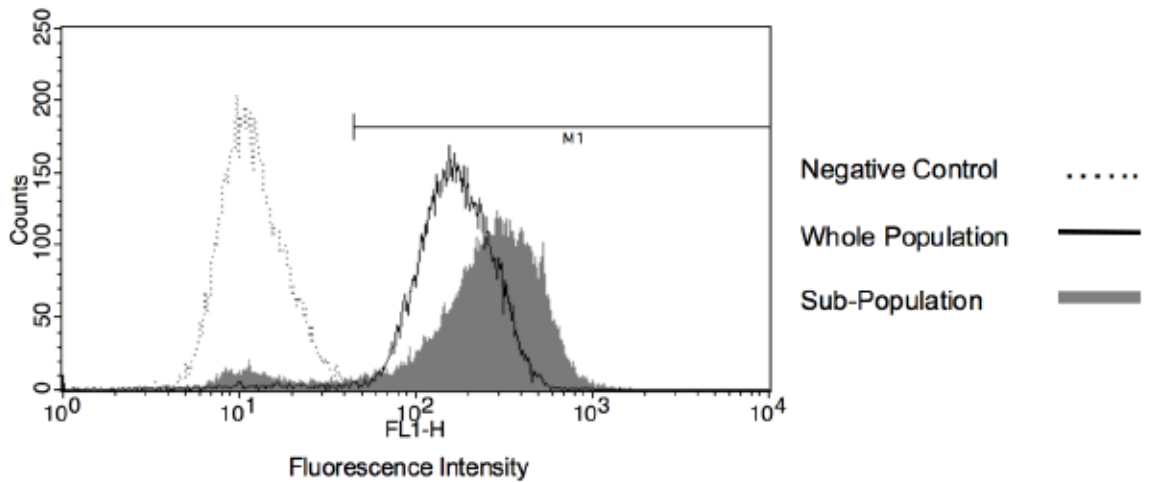
The objective of specific aim #5 was to determine if there was a difference in osteogenic capacity between MPCs and a specific subpopulation of cells in which the highly myogenic cells were removed. In order to do this the subpopulation of cells first had to be isolated based on methods described in section 3.3.6. Next, Pax7 expression was assessed in this isolated subpopulation. Because Pax7 is a marker of satellite cells it was chosen to observe if there were any differences between normal MPCs and the proposed subpopulation.

4.6.1 Assessing Pax7 expression in subpopulation of cells

Once the desired subpopulation of cells were successfully isolated, Pax7 expression was assessed using flow cytometry as per section 3.3.6. Figure 21.A depicts a general overlay of each of the two populations of MPCs compared to a negative control (not subject to Pax7 immunofluorescence). As viewed in the figure, there is a right-shift in fluorescence intensity in the subpopulation of cells compared to the normal or "whole" population of cells. This indicates that this subpopulation of cells are expressing more Pax7. Figure 21.B illustrates the purity of this subpopulation of cells based on the percentage of cells that were Pax7 positive within the desired gated region. As viewed in the figure, when compared to the whole population of cells the subpopulation of MPCs are 93.25% Pax7 positive while the whole population cells are 98.09% Pax7 positive. The differences observed between the two populations of cells as well as their

Pax7 expression relative to the negative control are statistically significantly different ($p \leq 0.05$), however the magnitude of these differences may not reflect a functionally relevant difference.

A



B

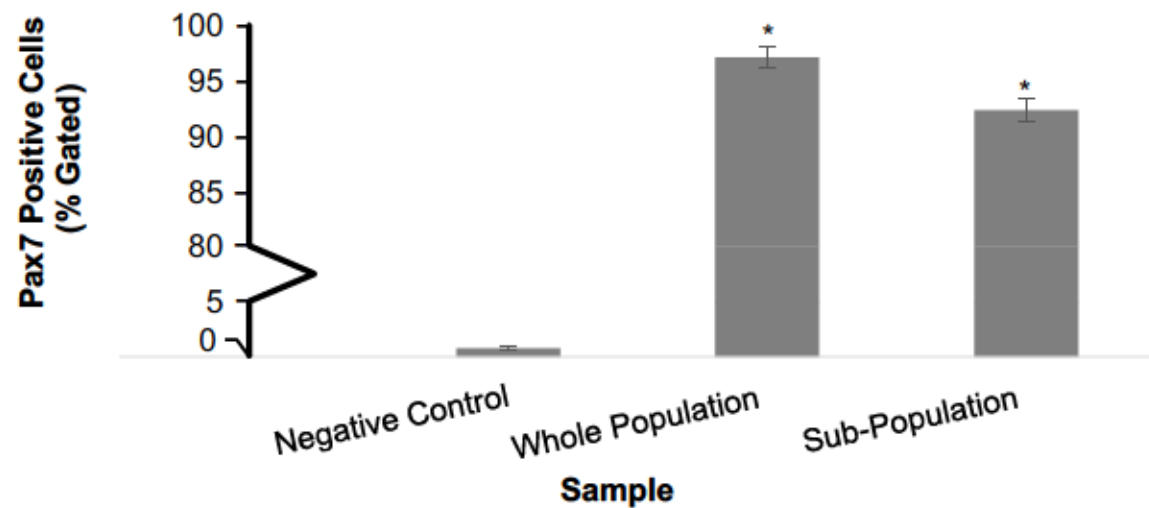


Figure 21. **Pax7 assessment of a subpopulation of MPCs utilizing immunofluorescence and flow cytometry.** In order to assess if there were any difference between MPCs (muscle precursor cells) and a subpopulation of MPCs in which the highly myogenic cells had been removed, the subpopulation was first isolated, then subjected to Pax7 immunofluorescence. Figure 21.A depicts a

general overlay of the negative control cells which were not subjected to Pax7 immunofluorescence (dotted line), as well as the normal MPC population or “whole population” (solid black line), and the subpopulation (grey shaded region). The M1 line represents the population of cells that are positive for the Pax7 marker. Figure 21.B depicts group mean data of which percentage of cells are Pax7⁺ in terms of mean \pm SEM (n=4). A break in the y-axis is denoted by >, and significant differences from negative control are denoted by *, (p \leq 0.05).

After determining the percentage of Pax7⁺ cells, the relative expression of Pax7 within the cells was analyzed as well. This was determined using mean, geometric (geo mean), and median (Table 2) fluorescence intensity. It was found that both the whole population and subpopulation of cells were significantly higher than the negative control in terms of Pax7 fluorescence intensity (p \leq 0.05). There were also significant differences observed between the whole population and subpopulation of cells in terms of Pax7 content (p \leq 0.05). The subpopulation of MPCs expressed more Pax7.

Mean Fluorescence Intensity			
Population	Mean \pm SEM	Geo Mean \pm SEM	Median \pm SEM
Negative Control	94.4825 \pm 10.96	76.7025 \pm 8.45	62.4275 \pm 7.69
Whole Population	214.695 \pm 15.35*	192.04 \pm 13.00*	192.05 \pm 13.52*
Subpopulation	375.875 \pm 52.55*	331.8175 \pm 46.97*	350.5825 \pm 49.46*

Table 2. **Assessment of mean fluorescence intensity of Pax7 immunofluorescence in the whole population versus a subpopulation of MPCs.** In order to assess the differences in Pax7 between the whole population of MPCs (muscle precursor cells) and a specific subpopulation, after the cells had been isolated and subjected to Pax7 immunofluorescence, data were analyzed in terms of mean, geo mean, and median. Data are expressed in terms of mean \pm SEM (n=4). These data represent the Pax7 protein content in the

cells. In respect to the cells expressing Pax7, Pax7 content was highest in the subpopulation of MPCs. Significant differences from negative control are denoted by *, ($p \leq 0.05$).

4.6.2 Assessing osteogenic capacity in subpopulation of cells

After determining the Pax7 expression and content the osteogenic capacity of the proposed subpopulation of cells was assessed. Cells were isolated and then re-plated and treated with 10ng/ml BMP-2 as per section 3.3.6. There were significant differences in ALP activity between the normal and subpopulations of cells after receiving the 10ng/ml BMP-2 dose ($p \leq 0.05$), however there was no effect observed between the 10ng/ml BMP-2 treatments and their respective control. Therefore, no conclusions can be made regarding any differences in terms of osteogenic capacity between the normal population and the subpopulation of MPCs.

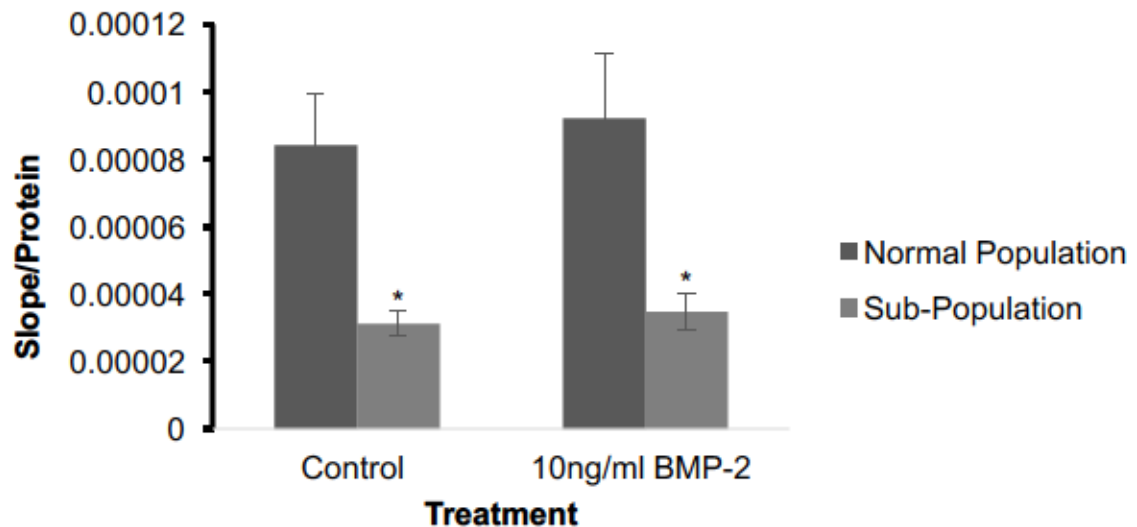


Figure 22. **Osteogenic capacity of the subpopulation of MPCs.** In order to assess if there were differences in osteogenic ability between the normal population of MPCs (muscle precursor cells) and the proposed isolated subpopulation, cells were grown then strained and re-plated. They were then

treated with 0 or 10ng/ml BMP-2 (bone-morphogenetic protein-2) and allowed to differentiate for 2-days. After 2-days of differentiation an alkaline phosphatase (ALP) activity assay was performed to assess osteogenesis. Data are expressed in terms of slope/protein of mean \pm SEM (n=6). Significant differences from the normal population are denoted by *, ($p \leq 0.05$).

Chapter 5. Discussion

In this investigation, the efficacy of ascorbic acid as a potential preventative treatment for HO was examined. Literature suggests that the cells involved in HO are MSCs which have migrated to the injury site following some sort of extreme trauma or surgery. Properties of MPCs, including their classification as a stem cell, suggests that they have the potential to be induced into an osteogenic lineage just pre-osteoblastic cell lines or MSCs do. This was confirmed during this study by inducing MPCs to undergo osteogenic differentiation using BMP-2. Following this treatment, MPCs demonstrated a significant increase in ALP activity ($p \leq 0.05$). When ascorbic acid was then introduced in addition to the BMP-2 treatment, there was no increase in ALP activity observed, indicating ascorbic acid had a blocking effect on the BMP-2 treatment. The mechanism of this action was then further assessed to determine if the actions of ascorbic acid were due to pro-oxidant activity. It was found that ascorbic acid does function as a pro-oxidant at the proposed doses used, indicated by significant increases in superoxide production detected by NBT assays ($p \leq 0.05$), and that these pro-oxidant effects can be reversed upon addition of an antioxidant.

Stem cells and progenitor cells, including MPCs, can be characterized in terms of molecular markers. These markers allow for tracking of the cell in its anatomical niche within a tissue. In adult skeletal muscle, the majority of MPCs express Pax7. Pax7 is a paired box transcription factor which is a defining characteristic of MPCs because it is specifically expressed in all quiescent and proliferating MPCs [158]. Therefore, this marker was chosen to assess if the cells utilized for this study were a pure population of MPCs. As viewed in Figure 10, 98.09% of cells subject to Pax7 immunofluorescence were Pax7⁺. This indicates the high level of purity of the MPCs used for this study.

5.1 Specific aim #1

5.1.1 Induction of MPCs to an osteogenic lineage

After determining if the cells utilized for this study were a pure MPC population, it had to be determined if they could be induced to undergo osteogenesis with BMP-2. This was achieved by treating the MPCs with a dose of 100ng/ml BMP-2 and measuring ALP activity. ALP activity has been routinely used in the literature and shown to increase with bone formation therefore it was an appropriate choice when choosing a marker for bone formation in this study. As viewed in Figure 11, treatment of MPCs with BMP-2 resulted in a significant ~2-fold increase in ALP activity ($p \leq 0.05$). This indicates that MPCs have the ability to undergo osteogenic differentiation. These findings are consistent with a number of investigations which demonstrate that when myoblasts are treated with BMP-2 or BMP-4, gene expression of osteogenic differentiation markers, such as ALP and osteocalcin, are increased, while markers of the myogenic

phenotype are terminated [81], [23], [166]. The maintenance of the osteogenic phenotype and repression of the myogenic phenotype however has been suggested to be dependent on the continual presence of BMPs [23]. Asakura *et al.* have demonstrated the presence of osteogenic cells in single muscle fiber cultures which they conclude were of satellite cell origin [81]. In contrast, Starkey *et al.* suggest that satellite cells from young adult mice are committed to myogenesis and do not spontaneously adopt non-myogenic fates such as osteogenesis in culture. They provide evidence that the non-myogenic phenotypes observed in MPCs are due to improper purification of the heterogeneous population [167]. Other studies have demonstrated that MPCs are capable of complete execution of adipogenic differentiation in addition to myogenic differentiation [168]. These researchers conclude that MPCs are capable of entering a mesenchymal alternative differentiation (MAD) program. This program demonstrates that MPCs preserve a mesenchymal repertoire which allows them to differentiate into a variety of different cell types. Our study reflects these findings that MPCs are capable of adopting other differentiation lineages including bone. A particular study supports these findings by identifying a population of myogenic progenitor cells which appear to be similar to the MPCs utilized for this study. These researchers report that myogenic progenitor cells spontaneously expressed both a marker of muscle satellite cells (Pax7) and a marker of osteoblasts (ALP) in regenerating human muscle. They report that these cells were able to undergo terminal osteogenic differentiation without exposure to an exogenous inductive agent such as BMPs while expressing the

muscle-lineage specific proteins Pax7 and MyoD [169]. In conclusion, their data suggest that human myogenic progenitor cells have the ability to act as osteoprogenitor cells which spontaneously form ectopic bone. This group also previously identified that mouse muscle satellite cells preserve the ability to undergo osteogenic terminal differentiation in vitro when BMPs were introduced. They suggest that perhaps failed restriction of phenotype plasticity may trigger ectopic ossification in muscle satellite cells [166]. Despite literature findings however, MPCs are still overlooked as a potential source of HO.

In addition to ALP activity, the osteogenic potential of MPCs was evaluated using a colorimetric quantification of calcium deposits method utilizing ARS. ARS is commonly used to identify mineralization based on the presence of calcium deposits. Typically, mineralization is observed and assessed after roughly 14-21 days [170], [171]. At this time, the cells are able to be stained with ARS and any formed calcium deposits will appear red. Unfortunately, MPCs are only able to be cultured with the ascorbic acid treatment for approximately 5-6 days before they become too confluent and will detach from the plate. This could perhaps be due to changes in their morphology as they begin to undergo the differentiation program. As such, when ARS was introduced to the cells sufficient mineralization had not occurred by day 5. To attempt to compensate for this the ARS was quantified using 10% CPC. As viewed in Figure 12, after treatment with 10ng/ml BMP-2, there was not a significant increase in ARS concentration observed between control and the BMP-2 treatment. This indicates that there may not have been enough time for mineralization to occur for this particular

assay. Alternatively, future studies could be performed with a higher dose of BMP-2 such as 100ng/ml. The higher dose may be sufficient to cause mineralization within a shorter time frame. Additionally, other markers of bone formation could be assessed as well such as osteocalcin.

BMP-2 has been utilized during *in vivo* studies as a model to study HO. During these studies, BMP-2 and MG is injected intramuscularly into an animal. This eventually results in formation of HO [149]. Although these studies are not an accurate representation of HO, they are useful as a model. Literature suggests that the cells responsible for forming HO are MSCs which have migrated to the injury site following a stimulus, such as trauma or surgery. If HO can be somewhat replicated by implanting BMP-2 into the region, that would indicate that BMP-2 implantation causes migration of MSCs to the region or injury site. BMP-2 is essential for the initiation of fracture healing and is responsible for regulating the recruitment of osteoprogenitor cells and activating osteogenic lineage differentiation during endochondral ossification. Mice who have been treated with BMP-2 after fracture show enhanced chondrogenesis [172]. However, migration of MSCs is likely not caused by BMP implantation. During normal fracture healing, bone is formed via endochondral and intramembranous ossification. Upon injury, inflammatory cytokines will be produced which recruit MSCs into the injury site. These MSCs will then proliferate into chondrocytes which will then undergo mineralization and eventually lead to mature bone formation [173]. Although BMP-2 implantation leads to ectopic bone formation, it may not necessarily cause migration of

osteoprogenitor cells to the injury site, a potentially more likely source of cells for HO are the MPCs which are already present within the muscle compartment. There would be no need for them to migrate to the site of injury as they reside within the muscle compartment already. This study demonstrates that MPCs have the potential to be induced into an osteogenic lineage just as literature demonstrates MSCs do with BMP-2.

5.2 Specific aim #2

5.2.1 Examining the effects of ascorbic acid on the osteogenic potential of MPCs

Once it was determined that MPCs were capable of undergoing osteogenic differentiation, the effects of ascorbic acid on osteogenesis were evaluated. This was done in order to assess the potential of ascorbic acid to be a new prophylactic treatment for HO. As viewed in Figure 13, when 100 μ M ascorbic acid was introduced in combination with the 100ng/ml BMP-2 treatment, there were no increases in ALP activity observed. This indicates that ascorbic acid has the ability to block the osteogenic effects of BMP-2 on MPCs. Due to complications with culturing conditions, described in section 5.1, there were no effects observed in Figure 14 when ARS quantification was assessed.

Ascorbic acid is known to stimulate the overall process of osteoblast differentiation including the expression of ALP, a marker for osteoblast formation [174], [175], [176]. It is important to note however, that this occurs at a much lower dose than described in this study and in normal bone-forming cell types. Ascorbic acid is an essential factor in normal osteogenesis. It promotes synthesis of type 1 collagen, interactions with integrins, activation of the protein kinase

pathway and phosphorylation of osteoblast-specific transcription factors [177]. It is a co-factor for enzymes which are responsible for hydroxylating proline and lysine in pro-collagen. Without ascorbic acid, proline is not hydroxylated and collagen chains are unable to form the proper helical structure. As such, the predominant role of ascorbic acid during osteogenic differentiation involves the secretion of collagen type 1 into the extracellular matrix (ECM) [178]. Collagen is a precursor of bone matrix mineralization. Osteogenic differentiation is frequently induced using a triad known as DAG consisting of dexamethasone, ascorbic acid and β -glycerophosphate. As such, the osteogenic blocking effects observed in MPCs by ascorbic acid should not be a concern in terms of normal bone repair at the injury or surrounding sites.

5.3 Specific aim #3

5.3.1 Examining the pro-oxidant potential of ascorbic acid

After determining that ascorbic acid had a blocking effect on BMP-2-induced osteogenesis of MPCs, the mechanism of these actions were evaluated. Within the body are ROS which are involved in normal biochemical processes including control of cell proliferation and cell signaling. These ROS however also cause cellular damage to DNA, proteins and lipids. As such, the body has antioxidant defense mechanisms in place to protect against harmful ROS. These defense mechanisms include enzymes which remove ROS such as superoxide dismutase, catalase, and peroxidase. They also include proteins which will sequester transition metal ions such as ferritin or transferrin. A final defense by

the body includes low molecular weight peptides and cofactors such as glutathione, NADPH and thioredoxin, and both lipid- and water-soluble dietary agents which scavenge ROS and nitrogen species such as vitamin E, vitamin C, and β -carotene [88]. As such, at physiological doses, ascorbic acid functions as an antioxidant. However, it is also known to function as a pro-oxidant at high physiological doses, resulting in oxidative damage. This is believed to occur by the reduction of ferric Fe^{3+} to ferrous Fe^{2+} ions (also Cu^{2+} to Cu^{1+}), which will then reduce H_2O_2 to hydroxyl radicals.

As described previously, ionization radiation therapy is a current common prophylactic treatment for HO. This therapy works by increasing the production of ROS or free radicals which cause oxidative stress in the individual. This oxidative stress is then able to disrupt the mechanisms involved with HO. As viewed in Figure 15, when MPCs were treated with a $200\mu\text{M}$ dose of ascorbic acid, superoxide production significantly increased ($p \leq 0.05$). This indicates pro-oxidant ability of ascorbic acid. In order to confirm these findings, antioxidants were added, in addition to ascorbic acid, to the MPCs. TEMPOL is an antioxidant that promotes the metabolism of superoxide and is therefore considered a SOD mimic. Based on these principles it could be effectively used to scavenge superoxide. PEG-Cat on the other hand was used to determine if a reduction in H_2O_2 would reverse any potential effects observed by ascorbic acid. When 1mM TEMPOL was introduced in addition to the $200\mu\text{M}$ ascorbic acid treatment, there was no increase in superoxide production observed. This indicates that TEMPOL successfully scavenged any ROS (perhaps superoxide) produced by ascorbic

acid. Conversely, when 200U PEG-Cat was introduced in combination with 200 μ M ascorbic acid, superoxide production remained elevated. This indicates that ascorbic acid is likely not exerting pro-oxidant actions through the production of H₂O₂ at this dose.

These findings are consistent with a number of studies which identify pro-oxidant potential of ascorbic acid [95]. Many of these studies have been reviewed by Carr and Frei [15]. It is based on this principle that ascorbic acid has been investigated as a potential cancer treatment. One of these investigations involves using ascorbic acid in combination with chemotherapy to treat pancreatic cancer [179]. Pharmacologic concentrations have also been investigated as a pro-drug for H₂O₂ delivery to tissues to selectively kill cancer cells [101]. The use of high-dose ascorbic acid therapy in the treatment of patients with advanced cancer has been extensively reviewed by Ohno and colleagues [180]. It has been suggested that the ascorbic acid-mediated Fenton reactions, which allow ascorbic acid to function as a pro-oxidant, should be controlled in the human body by efficient iron sequestration by metal binding proteins such as ferritin and transferrin. As such, there have been arguments made that the pro-oxidant effect may not be relevant *in vivo* [181], [15]. As discussed above however, many *in vivo* studies have been conducted based on pro-oxidant potential of ascorbic acid with promising results. Additionally, an alternative mechanism has been proposed in which ascorbic acid functions as a pro-oxidant in the absence of transition metals. This study suggests that ascorbic acid induces the decomposition of lipid hydroperoxides to genotoxic bifunctional electrophiles *in vivo* without the requirement for transition

metals [182]. Transition metals are present in many buffer solutions and other components of cell culture reagents. Therefore, *in vitro*, lower doses of ascorbic acid are likely needed to produce a pro-oxidant response. If ascorbic acid were to be investigated *in vivo* in terms of pro-oxidant potential, a higher dose than what is suggested in this study would likely be needed.

5.3.2 Examining the pro-oxidant effects of ascorbic acid on BMP-2 treated MPCs

In order to assess if the blocking effects of ascorbic acid on BMP-2 were due to pro-oxidant activity, ALP activity assays were performed. As viewed in Figure 16, when 100 μ M ascorbic acid was added in combination with the 100ng/ml BMP-2 treatment, there were no increases in ALP activity observed. Following the addition of antioxidants to these two treatments, 1mM TEMPOL successfully blocked the BMP-2-inhibiting effects of ascorbic acid and ALP activity significantly increased ($p \leq 0.05$), compared to the 100ng/ml BMP-2 + 100 μ M ascorbic acid treatment. In contrast, when PEG-Cat was added in combination to ascorbic acid and BMP-2, ALP activity did not increase. This indicates that PEG-Cat was not able to block the effects of ascorbic acid on BMP-2-induced osteogenesis of MPCs. In summary, TEMPOL successfully blocked the effect of ascorbic acid on BMP-2-induced osteogenesis and PEG-Cat did not. This suggests that ascorbic acid likely blocks BMP-2 by acting as a pro-oxidant via the production of superoxide. Therefore, increased doses of ascorbic acid could theoretically function in a similar manor to ionizing radiation therapy and, by producing oxidative stress in the individual, disrupt the mechanisms associated with HO. As discussed previously however, for *in vivo*

studies, a larger dose of ascorbic acid than those proposed in this study would likely be needed. The doses of ascorbic acid proposed for this study likely result in successful pro-oxidant activity due to the presence of transition metals in culture buffers and reagents. In addition, using ascorbic acid to prevent HO versus radiation therapy or NSAIDs minimizes risk factors and harmful side-effects. There have been a few hypothetical adverse effects cited in literature regarding high doses of ascorbic acid [183], [184], however most of these, with the exception of minor gastrointestinal effects, have not been exclusively proven.

5.4 Specific aim #4

5.4.1 Examining effects of ascorbic acid on myogenic ability of MPCs

One of the major risk factors associated with the current preventative treatments available for HO is the potential for nonunion of bone. The effectiveness of a treatment is important, however so is not impairing normal regeneration and healing of bone and muscle or other tissues at the injury site. As such, it is important when considering alternative treatments that normal healing processes are not affected.

Skeletal muscle contains approximately 40% of whole-body ascorbic acid content. In muscle cells, ascorbic acid protects cells against oxidative stress [164]. Additionally, ascorbic acid functions within muscle to maintain the activity of ϵ -N-trimethyllysine hydroxylase. This enzyme is required for carnitine biosynthesis, which is an important energy producing component of skeletal and cardiac muscle [165]. As such, it was hypothesized that ascorbic acid would not

have any negative effects on myogenesis however this had to be fully examined to be confirmed. Specific aim #4 was designed to investigate the effects of ascorbic acid on both the myogenic capacity and the proliferation of MPCs. To compare culturing conditions, MPCs were cultured in both DM and GM. DM is the most common method utilized to differentiate cell types and is known to work better because the lower serum concentration forces the cells into their differentiation program. Culturing cells in GM was done in order to investigate the effects of ascorbic acid on differentiation under less favorable conditions. As viewed in Figure 17, there were no detectable differences observed between myotube formation of MPCs between control and cells treated with 100 μ M ascorbic acid. The same trend was observed with cells cultured in both DM and GM, indicating ascorbic acid did not interfere with formation of mature myotubes.

Myosin heavy chain and muscle creatine kinase are two proteins specifically expressed in muscle. As such, they were chosen to evaluate myogenesis of MPCs. Myosin heavy chain was investigated using western blots and protein assays. As viewed in Figure 18, MPCs were cultured in both GM and DM to compare the effects of ascorbic acid under different cell culture conditions. Figure 18.A displays MPCs cultured in GM. A representative western blot is displayed showing treatment with varying doses of ascorbic acid (50 μ M, 100 μ M or 150 μ M). This blot represents myosin heavy chain protein content based on band density. When cells were cultured in GM they were still forced to differentiate, due to confluency, however not nearly as well as when they were cultured in DM. This is illustrated in the figure, based on the density of the bands,

under both culturing conditions. Also in Figure 18.A, is group mean data of western blots performed with varying doses of ascorbic acid (50 μ M, 100 μ M or 150 μ M). There were no differences observed between control and any of the doses of ascorbic acid tested, indicating no effect on differentiation. Figure 18.B displays the same trends however in MPCs cultured in DM. When density was quantified in terms of intensity/mm² and group mean data was represented in terms of fold difference of means, there were no significant differences found in myosin heavy chain protein expression between control and ascorbic acid treatments. Together, these results indicate that ascorbic acid has no effect on myosin heavy chain protein, and therefore likely no effect on normal myogenesis, in both favorable and unfavorable differentiation conditions. These findings are consistent with other reports which suggest that ascorbic acid stimulates the differentiation of smooth muscle cells. One particular report found that ascorbic acid stimulated the production of smooth muscle-specific myosin heavy chain-1 in rat cultured vascular smooth muscle cells [185].

Similar results were observed following transfection of MPCs with muscle creatine kinase promoter plasmids using the dual-luciferase reporter assay system. As viewed in Figure 19, when cells were differentiated in both GM and DM, there were no statistically significant differences found between control and any of the doses of ascorbic acid (50 μ M, 100 μ M or 150 μ M). This indicates no effect on muscle creatine kinase and therefore likely no effect on myogenic capability of MPCs or overall myogenesis. These findings were not surprising because in skeletal muscle, ascorbic acid is responsible for enhancing carnitine

biosynthesis. It is also utilized by the muscle to protect cells against ROS generated by physical exercise. *In vivo*, ascorbic acid is found at physiological concentrations and functions as an antioxidant, however it should be noted that there are less transition metals present *in vivo* compared to *in vitro* cell culture conditions. Skeletal muscle is able to take up the water-soluble ascorbic acid from the extracellular environment and recycle it intracellularly to maintain high cellular stores of ascorbic acid within the muscle [164]. Mean plasma ascorbic acid levels are typically between 50-60 μ M in healthy, well-nourished, non-smoking individuals. With oral supplementation and long-term vegetarian diets, these levels can be increased to roughly 100 μ M [186]. Plasma levels of ascorbic acid are not observed to be higher than 100 μ M, even with supplement doses approaching above 500mg/day because it is efficiently excreted in the urine [109]. Studies have reported that increases in plasma ascorbic acid results in increase in intracellular levels of the vitamin [187]. These increases however are likely due to cellular saturation and are often not dose-dependent [188]. As such, intracellular ascorbic acid concentrations of neutrophils, monocytes and lymphocytes saturate at lower supplementation doses compared to human plasma [109]. These literature findings support the differences between *in vivo* and *in vitro* studies. Although the doses proposed in this study were high enough to cause ascorbic acid to function as a pro-oxidant, without affecting myogenic processes, the same may not be true of *in vivo* testing. As a result, *in vivo* trials would need to be conducted to determine at which dose ascorbic acid exerts its effects optimally. Our findings support, in conclusion, that at increased

physiological doses, when ascorbic acid functions as a pro-oxidant, there are no negative effects on myogenesis of MPCs.

5.4.2 Examining effects of ascorbic acid on proliferation of MPCs

In order to assess if ascorbic acid had any effect on proliferation of MPCs, BrdU incorporation was utilized. This method uses pulse-labelling techniques to label the cells in the S-phase of the cell cycle with BrdU. The BrdU that is successfully incorporated into cellular DNA, during proliferation, is then detectable using flow cytometry. This allows for assessment of cell proliferation. Figure 20.A displays a representative histogram of MPCs treated with 100 μ M of ascorbic acid. The M1 line represents all proliferating cells which are BrdU positive in the defined gated region. As viewed in the histogram, there were negligible differences, between the control and the 100 μ M ascorbic acid treatment, in terms of the amount of BrdU positive cells. Group mean data were further represented in Figure 20.B. There were no differences between control and any of the doses of ascorbic acid with the exception of the 500 μ M dose. There was a significant difference between control and the 500 μ M ascorbic acid treatment ($p \leq 0.05$), indicating a negative effect on proliferation. However, at this dose there was also significant cell loss so this interpretation may not be entirely accurate. These results are consistent with another report in which cultured MSCs were supplemented with 250 μ M and 500 μ M ascorbate-2-phosphate (a stable derivative of ascorbic acid). They found increased proliferation activity with the 250 μ M dose and decrease rate of cell proliferation with the 500 μ M dose. They suggest the 500 μ M dose may have induced cellular damage during culture

[189]. It is important to note these were different cells however than the MPCs utilized for this study. Other studies report that millimolar, or non-physiological, doses of ascorbic acid in human hepatoma cells resulted in re-differentiation and growth inhibition. It was suggested that this was due to increase in H_2O_2 content within the cells [190]. These results were not surprising because while ascorbic acid is commonly added to cell culture medium at specific concentrations to increase proliferation and DNA synthesis of cells, at certain high concentrations it may be cytotoxic to cells. Ascorbic acid is well-known to stimulate the proliferation of various mesenchymal-derived cell types such as osteoblasts, adipocytes, chondrocytes, and odontoblasts [191], [185], [192], [177], and to modulate cell proliferation *in vitro* [193], [194, p. 19], [191]. Reports of the effects of ascorbic acid on cultured cells however are conflicting, some demonstrating inhibition of cell death by ascorbic acid while others suggest ascorbic acid is cytotoxic. Clément and colleagues report that the toxicity of ascorbic acid (ascorbate) is due to ascorbate-mediated production of H_2O_2 and the extent of which depends on the type of culture medium used. They report that 1mM of ascorbic acid generated $161 \pm 39 \mu M H_2O_2$ in DMEM and induced apoptosis in 50% of HL60 cells. Conversely, when RPMI 1640 medium was used, only $83 \pm 17 \mu M H_2O_2$ was produced and no apoptosis was detected [195]. Their results suggest that ascorbic acid itself is not toxic to the cells rather it is the ability of ascorbic acid to interact with different cell culture media to produce H_2O_2 at different rates. It is thought that this production of H_2O_2 is due to presence of trace levels of contaminant catalytic transition metals present in most salt and

buffer solutions involved with research [96]. In conclusion, our findings support that ascorbic acid did not affect differentiation or proliferation of MPCs with the exception of the 500 μ M dose.

5.5 Specific aim #5

The basic cellular unit of adult skeletal muscle is the myofiber. These myofibers are repaired and regenerated by satellite cells. As discussed, satellite cells reside beneath the basal lamina surrounding each fiber. They provide a source of new myonuclei for growing myofibers before they become quiescent in mature muscle. A key feature of these cells are their ability to proliferate and differentiate in response to muscle injury or trauma for muscle regeneration, while also undergoing self-renewal to replenish the cell pool. This means they are capable of asymmetric cell division [196]. Quiescent satellite cells express the paired box transcription factor Pax7. Once they become activated, they will co-express Pax7 along with MyoD, a transcription factor involved in myogenic differentiation. Some of the activated cells (now the progeny of satellite cells, termed MPCs) will then proliferate, downregulate Pax7, then differentiate. Other proliferating myoblasts will maintain Pax7 but downregulate MyoD in order to self-renew or re-enter a quiescent state [196], [7]. Therefore, cells which express more Pax7 are more likely to undergo self-renewal. It has been demonstrated that a specific pathway known as the Notch pathway is responsible for upregulating Pax7 and promoting satellite cell self-renewal [197].

5.5.1 Examining Pax7 expression in a specific subpopulation of MPCs

The goal of specific aim# 5 was to determine if there was a difference in osteogenic capacity between MPCs and a subpopulation of cells where the highly myogenic cells had been removed. This subpopulation of cells was isolated as per 3.3.6.1. After isolation, flow cytometry studies were performed using Pax7 immunofluorescence to determine if this population of cells displayed differences in Pax7 content. As viewed in Figure 21.A, there is a clear right-shift in fluorescence intensity in the subpopulation of cells versus the normal or “whole population”. The right-shift indicates that this subpopulation of cells is expressing more Pax7. Wen and colleagues suggest that cells expressing more Pax7 are more indicative of a more primitive stem cell lineage [197]. As such this subpopulation of cells may be more likely to self-renew, rather than enter the differentiation program. Figure 21.B illustrates that a significantly larger percentage of the “whole population” cells are expressing the marker Pax7 ($p \leq 0.05$), however Figure 21.A and Table 2 suggests that of these cells expressing Pax7, the subpopulation group are expressing more Pax7, compared to the whole population. Table 2 illustrates the overall Pax7 content observed in the two populations of MPCs based on mean, geo mean (geometric mean) and median fluorescence intensity. Each flow data measurement provided similar results however some are more accurate than others. Mean fluorescence intensity cannot be used to generalize a population of events, because a right-handed skew causes an exaggeration of the mean. In order to compensate for this, a geo mean is used to account for the log-normal behavior of flow data. The

geo mean however is also susceptible to significant shifts. Therefore, the most accurate measurement is the median. Median fluorescence intensity is a robust statistic which is less influenced by skew or outliers. It is therefore the safest choice and most accurately represents a typical cell. In terms of median fluorescence intensity, both the whole population and subpopulation of cells differed significantly from negative control ($p \leq 0.05$). Additionally, the whole population and subpopulation significantly differed from one another ($p \leq 0.05$). These results were also observed for mean and geo mean data. The heterogeneity of MPCs is further supported by other groups which report the presence of subpopulations of satellite cells which are distinct in phenotype and function. They suggest that satellite cells are a heterogeneous population composed of stem cells and committed myogenic progenitors [198]. Other studies involving radioisotope labeling of growing rat muscle identify that satellite cells are a mixture of 80% fast-cycling cells and 20% slow-cycling “reserve cells” [82]. Beauchamp and colleagues suggest that satellite cells exhibit heterogeneous expression of satellite cell markers CD34, M-cadherin, and *Myf5-nLacZ* also [199, p. 34], and Montarras and colleagues demonstrate that a portion of satellite cells have the capacity to repopulate the satellite cell pool as well as contribute to regenerating muscle [200].

5.5.2 Examining if the subpopulation of MPCs have increased osteogenic capability compared to the normal population of MPCs

Originally it was hypothesized that the subpopulation of cells would be less likely to undergo myogenesis and more likely to undergo osteogenesis. It

was thought, that because they may be more likely to undergo self-renewal, they may more resemble a primitive stem cell that can be influenced to undergo different types of differentiation. Based on the results in Figure. 23 however, there was no indication that these cells were more likely to undergo osteogenesis when induced with BMP-2 than the normal MPC population. Rather, the opposite results were found, ALP activity was significantly higher in the normal population versus the subpopulation of cells. Interestingly, the subpopulation of cells expressed more Pax7 overall, as viewed in Table 2. This may indicate that a more primitive stem cell will be less likely to be driven into an osteogenic lineage. Rather than it being the cell that is more likely to self-renew, it may be the cell that has already committed to differentiation, and is instead adopting a more osteogenic lineage rather than a myogenic lineage, that is responsible for HO.

Chapter 6. Conclusion

The current study was designed in order to assess if ascorbic acid could be used as a prophylactic treatment for HO. The model utilized for this study involved cell culture procedures with a specific population of cells known as MPCs. They are derived from a specific type of stem cell known as a satellite cell. This study demonstrates that MPCs have the potential to be induced to an osteoblastic lineage under the appropriate signaling environment, therefore making them a candidate for cells involved in HO formation. These cells are overlooked in the literature as being a potential source. Rather, a different population of cells known as MSCs are thought to be responsible. It is thought

that these MSCs will migrate to the injury site following trauma or surgery and, rather than adopting a myogenic lineage, they will instead adopt an osteogenic lineage. This study suggests a more likely cell source, MPCs, which are already present within the muscle compartment and have the ability to adopt osteogenic lineages, to be the main cells contributing to HO.

Ascorbic acid is a vitamin required for various biological functions. It is the most important water-soluble antioxidant in human plasma and mammalian cells. Humans, unlike most animals, are not capable of synthesizing this vitamin therefore it must be incorporated into their diet. Under normal physiological doses it functions as an antioxidant. However, we demonstrate in this study that, at increased doses, it functions as a pro-oxidant as well. Results from this study suggest that ascorbic acid acts to block the osteogenic effects observed by BMP-2 induction via production of ROS, specifically superoxide. In order to assess if ascorbic acid could be used as a successful prophylactic treatment for HO animal studies would need to be conducted followed by clinical trials. The cell culture model described in this study provides the preliminary foundation for future investigation, however much more work would need to be done in order to accurately examine the effects of ascorbic acid on HO prevention.

In summary, although there are current methods of prophylaxis available, an alternative preventative treatment for HO is needed. Both radiation therapy and the use of NSAIDs have harmful risk-factors and side-effects associated with them, and been developed for use in a civilian setting only. These options are not feasible or realistic in settings such as the military, where high impact trauma

such as blast injuries or combat injuries are prevalent. As discussed, HO has increased incidence in these type of settings versus the civilian population. Ascorbic acid induces oxidative stress by functioning as a pro-oxidant at high physiological concentrations. This property is being investigated in terms of cancer treatment however can also be applied to HO prevention. Ascorbic acid may provide an alternative safe, reliable, practical, and cost effective treatment for those who could potentially suffer from this condition. Preliminary work involves cell culture studies and the development of animal studies prior to developing clinical trials. If clinical trials are developed, the doses of ascorbic acid suggested in this study can be achieved via oral administration. They are developed for a cell culture model however and due to the involvement of transition metals in cell culture studies, a higher dose would likely be needed for *in vivo* trials. As previously described, a maximal oral dose of 3g of ascorbic acid daily results in plasma levels around 220 μ M [105]. Intracellular levels saturate much quicker however with a much smaller dose. They can reach levels approximately 14 times that of the plasma. Therefore, a dose significantly lower than the maximal dose of ascorbic acid acceptable would result in the levels proposed in this study. In summary, the ease of administration combined with minimal risk, low to zero toxicity, and lack of long-term, harmful side-effects, may make vitamin C an optimal choice in comparison to current prophylactic treatments for HO such as radiation therapy or the use of NSAIDs.

Chapter 7. Bibliography

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Chapter 8. Appendix

8.1 Cell Splitting Protocol: Lees Lab

- Obtain trypsin-based enzyme dissociation solution (trypsin for cell lines, TrpLE for primary cells –stored at room temperature) from freezer and thaw in bead bath (inverting often, volume needed depends on flask size in use)
- Prepare required amounts of media in sterile tubes and put in bead bath to warm
 - Enough to neutralize trypsin (double the volume of trypsin used)
 - Enough to seed back cells + 1 mL extra for pipetting error + 1 mL for re-suspension
 - Depending on flask size, enough media for new flask, leaving enough space (volume) to seed cells (warm in incubator)
- Remove microscope cover and clean stage with ethanol
- Obtain cells from incubator and check cell confluency and record
- Bring cells into BSC
- Aspirate media from flask with vacuum and Pasteur pipette
- Add 20 mL of sterile DPBS to the flask to wash cells. Gently agitate flask.
- Aspirate DPBS
- Add trypsin to flask and place cells in incubator until cells have detached (confirm with microscope)

- Bring neutralizing media from bead bath into BSC (clean first under cap with ethanol!)
- Add 180 μ L PBS to a 1.5 mL Eppendorf tube
- Bring cells into BSC
- In 25mL serological pipette obtain enough media to neutralize trypsin. Keeping media in pipette, remove trypsin from flask. Empty the trypsin portion in pipette into 50 mL sterile tube. Use the remaining media to wash the walls of the flask and take up all solution and add to 50 mL tube (final volume of media + trypsin)
- Balance tube on scale with another tube of equal weight
- Centrifuge at 150x g for 7 minutes (see Appendix 5.5 for other parameters depending on cell type), room temperature (program-8-enter-enter-start)
- Clean Hemacytometer by gently wiping with ethanol (device and coverslip)
- Open program on computer
 - Hemacytometer Template
 - Microsoft Word
- Bring in remaining media from bead bath (clean first with ethanol)
- Obtain cells from centrifuge, clean tube with ethanol gently, without disturbing the pellet
- Decant off supernatant into old flask, using vacuum to remove last drops from edge of tube
- Re-suspend pellet in 1 mL media (mix well with pipette)
- Take 20 μ L of cell suspension and add to 1.5 mL Eppendorf tube containing 180 μ L PBS (mix with the 200 μ L pipette)
- Take 10 μ L out of 1.5 mL Eppendorf tube and add to each side of the Hemacytometer (pipette directly under coverslip into groove)
- Using microscope, count all of the cells in each of the 9 squares on both sides of the device. Use the Microsoft Word document to keep track of your count, pressing a letter key each time you see a cell. Keep the counts from each side separate. You will average these. Record number in Hemacytometer Template and record cell count in cells/mL. The actual calculation is as follows: $(\text{cell count 1} + \text{cell count 2})/2 = x$. $x/9 = \text{cell count per square}$. $\text{Cell count per square} \times 10^4 \times 10 (\text{dilution factor}) = \text{cells/ml}$.
- Calculate what volume of cells are required to seed back into new flask(s). Cell number depends on experiments to be run, and a 50 000 cells/ml concentration is typically made up.
- Add appropriate volume of cells to media tube (same tube that you removed the 1 mL for re-suspension from), mix well with serological, then add the calculated volume of cell mixture to required flasks
- Incubate flasks at 37°C

8.2 Western Blot SOP: Lees Lab

Day 1 - Gel Preparation and Running

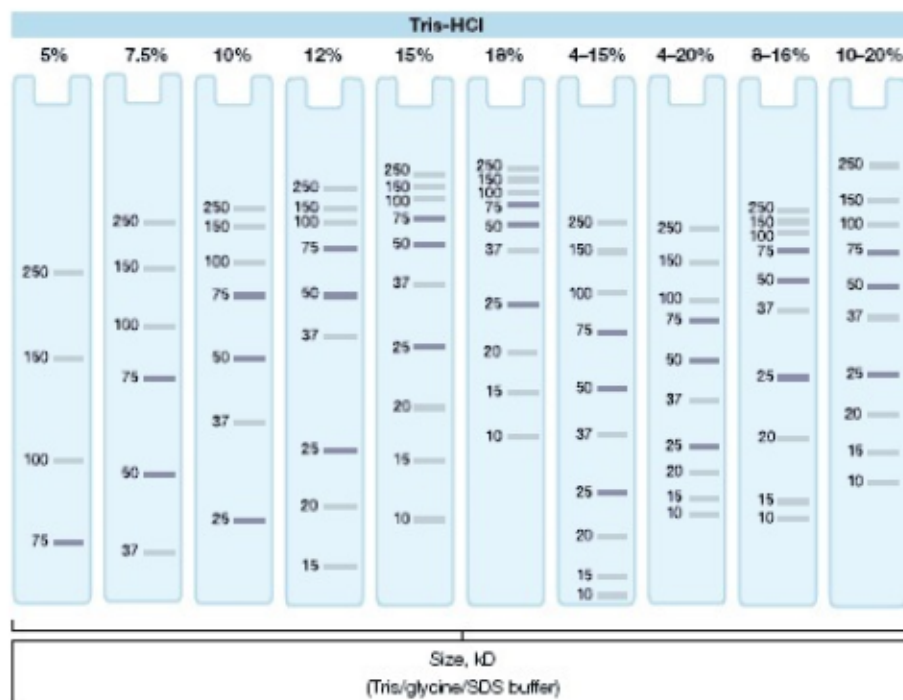
What you need:

- gel apparatus with sponges (bench)
- glass plates (2 sized (1.5mm), 2 short plates) (bench)
- 2 green plate holders (bench)
- combs same size as glass plates (bench)
- 2x 50mL beakers (bench)
- 1x 50mL tube
- 10ml serological pipettes
- 2 transfer pipettes
- Distilled water (DW)
- 1.5M Tris pH 8.8 (4°C)
- 0.5M Tris pH 6.8 (4°C)
- 10% SDS (bench)
- 40% acrylamide (4°C)
- Ammonium persulfate (APS, 4°C)
- TEMED (chemical storage cabinet)
- 20% methanol (bench)
- 0.1% SDS (bench)
- standard ladder (molecular marker) (-20°C)
- gel running apparatus and container (bench)
- 10ml syringe with needle

First remove samples from -80°C to thaw on ice. If a white precipitate is present after thawing, place samples at 37°C (using a heat block) until they are clear. This should only take a few minutes)

1. Obtain glass plates from drying rack on bench. If there is anything to clean off, use a kimwipe with DW
2. Place glass plates in green holders with the doors open, making sure both plates lay flush with the surface of the bench, and with each other. Next, while applying slight pressure to the tops of the glass plates, close the doors.
3. Place the well combs between the glass plates. Measure 11mm from the bottom of the well comb and place a mark. This is your pour line for your gel. Remove the combs and set aside.
4. Prepare 10% APS in a 1.5ml eppendorf tube: add 0.1g APS (kept at 4°C) to 1ml DW. Triturate until dissolved. Make fresh daily.
5. Prepare your separating gel in a 50ml beaker with a stir bar. Your gel percentage depends on the weight of your target protein. Use the chart below to choose the appropriate percentage of gel to make.

Migration Pattern for Ready Gel Tris-HCl Gels



<http://www.bio-rad.com/en-us/product/ready-gel-precast-gels/ready-gel-tris-hcl-precast-gels>

Volume (ml)

Stock Component	5%	7.5%	10%	12%	15%
Distilled Water	12.3	10.93	9.68	8.68	7.18
1.5M Tris, pH 8.8	5	5	5	5	5
10% SDS	0.2	0.2	0.2	0.2	0.2
40% Acrylamide	2.5	3.75	5	6	7.5

The percentage of acrylamide determines the percentage of gel you are making. So, if you have 30% acrylamide to start, you will need to adjust volumes accordingly. For example, for a 10% gel, you will need 6.67ml of 30% acrylamide, and 8.01ml of DW. The DW is to make up the final volume of the solution to ~20ml.

- Once the stock components are mixed for the appropriate separating gel percentage, place the glass plates that are in the green holders onto the sponges of the gel apparatus. Clip them in. Ensure they are sitting flush on the sponges.

7. ***See note below.** Place the beaker on a stir plate, mixing gently so as not to introduce bubbles. While mixing, quickly add 100ul 10% APS and 20ul TEMED. Allow to mix for 30 more seconds.
8. Using a transfer pipette, pipette gel mixture quickly between the plates, moving back and forth between the two sets of plates after each pipette-ful. Fill each set of plates to your marker line.
9. Carefully overlay the separating gel with 20% methanol using a syringe. Allow to polymerize for 30 mins. **Tip: leave your transfer pipette in your beaker containing left over gel solution. If this is polymerized after 30 minutes, your gel between the plates will be too.*
10. During this polymerization, mix your stock components for your stacking gel in a 50ml beaker containing a stir bar:

	Volume (ml)
Stock Component	4%
Distilled Water	12.68
0.5M Tris, pH 6.8	5
10% SDS	0.2
40% Acrylamide	2

Note the use of a different Tris buffer

11. Once the gel is polymerized, pour the methanol down the sink and rinse the empty area between the plates three times with 0.1% SDS in a syringe (keep gels on the apparatus during this time). Ensure all SDS is emptied from this area by tilting the apparatus to the side and holding kimwipes to the top edge of the glass plates.
12. Place the beaker on a stir plate, mixing gently. While mixing, quickly add 100ul 10% APS and 20ul TEMED. Allow to mix for 30 more seconds.
13. Using a transfer pipette, overlay the separating gel with the stacking gel solution, filling to the top of the plates. Insert comb on an angle slowly so as not to introduce bubbles or displace too much gel solution. Allow to polymerize for 30 mins

***Note:** If a vacuum degasser system is available, make up both the separating and stacking gel solutions in their beakers with stir bars (without the APS and TEMED), mix briefly on the stir plate and place both beakers into the degasser. Put the lid on and turn the vacuum pump on. Leave for 20 minutes to remove the air from the solutions. After 20 minutes, turn the pump off, remove the separating gel, and while gently mixing on the stir plate, continue as for step 7. While the separating gel is polymerizing, put the lid back on the degasser to protect the stacking gel.

14. Put your molecular marker (ladder) on ice
15. Prepare your 1X Running Buffer as described in the Buffers section. This can be prepared in advance and stored at 4°C
16. Once the gel is polymerized, remove the combs by pulling them straight up and out. Remove the glass plates carefully from the holders and place them onto the middle section of the apparatus (containing the electrodes). The short plates of each set should be facing each other. Place this section into the beige middle part with clear "doors". The doors should be open while the electrode is being inserted. Apply gentle downward pressure to the electrode section while closing the doors. Place this in the clear container.
17. Fill the middle section between the two gels with 1X Running buffer. Next, fill the clear container half way
18. Fill a Styrofoam box with ice. Create a spot to put the gel container. Place the gel container in this spot and push the ice against the sides of the container.
19. Begin loading your samples and ladder into the wells of the gel. You should load the wells of the gel closest to you first, and then turn the whole Styrofoam box to load the other gel.
Typically, 5ul of ladder is loaded into the first well on your left. All wells should be filled to ensure the samples run straight down.
20. Once all samples are loaded, place the lid onto the container (black to black electrode, red to red electrode). Plug the cords into the power supply and turn on. Turn the voltage up to 200V and press the button that looks like a man running. Make sure to observe bubbles in the running buffer, signifying the gel is running.
21. The samples are condensed into a solid blue line while they run through the stacking gel. This ensures that all samples enter the separating gel at the same time, and therefore have the same amount of time to run through the gel.
22. Allow your gel to run until the blue dye front completely runs off the bottom of the gel. This typically takes just over 1 hour. While this is happening, gather your transfer supplies and make fresh 1X Transfer Buffer, as per the Buffers section. This can be made in advance and stored at 4°C.

Gel Transfer

What you need:

- transfer apparatus and container (bench)
- 2x cassettes (bench)
- 2 plastic containers for soaking filter paper, sponges, and membranes (bench)
- 2 plastic containers for soaking the gels (bench)

- 4x black sponges (bench)
- 4x filter paper (bench)
- 2x nitrocellulose membrane (bench)
- flat forceps (bench)
- 1x transfer buffer (4°C)
- ice pack (-20°C)
- stir bar
- gel wedge

23. Once the gel is finished running, bring the entire container to the sink and dump out the running buffer. **Do not reuse this buffer.** Disassemble the apparatus to remove the glass plates.
24. Using the gel wedge, release the gel from the big plate so the gel is kept on the short plate. Cut the stacking gel off using the wedge and discard. Make a nick in the top left corner (usually the corner containing your ladder).
25. Add 1x Transfer buffer to a container. Place the short plate with the gel on it over the top of the container. By allowing the buffer to make contact with the gel, it should take the gel off of the plate itself. If this does not work, or if the gel stayed on the big plate rather than the short plate, use the wedge to gently lift the gel off of the plate and place into the buffer. Repeat with second gel in a separate plastic container.
26. Cut two membranes from the nitrocellulose roll using the filter paper as a size guide. Be careful not to touch the membrane with your gloves. Keep the blue paper on while cutting.
27. In another plastic container, place one sponge, one filter paper, one membrane, one filter paper, one sponge, and fill with 1X Transfer buffer. Repeat for second membrane.
28. Place all plastic containers on the belly dancer for 15 minutes, with slight agitation. This is necessary to equilibrate all components of the transfer "sandwich"
29. While these components are soaking, wash the running apparatus. To do this, re-assemble without the glass plates and fill the container with DW. Discard and repeat for a total of 3 times. Allow to dry on the drying rack or paper towel. **Do not hang.**
30. Assemble the sandwiches out of buffer on paper towel on the bench in the following order: clear side down, sponge, filter paper, membrane (move with forceps), gel (move with gel wedge, place so that cut corner remains on your left, ensure no bubbles), filter paper.
31. Use a 50ml tube to roll out any bubbles by starting in the middle of the filter paper and rolling outward. Repeat in opposite direction. Complete the

- sandwich by placing the second sponge onto the filter paper. Close the sandwich and repeat with the second one.
32. Place the sandwiches in the centre of the transfer apparatus with the black side of the cassettes facing the black side of the apparatus. Place the apparatus in the clear container (same one used for running of gel), add a stir bar, and place the ice pack in the unfilled space in the container.
 33. Fill the container up to the edge with fresh 1X Transfer buffer
 34. Move the apparatus to the clear door fridge onto the stir plate. Turn the stir plate on to low, making sure the stir bar moves easily. Place lid on top, red to red electrode and black to black electrode.
 35. Turn the power supply on to 30V and run overnight.

Day 2 – Ponceau S, Blocking, and Primary Antibody

What you need:

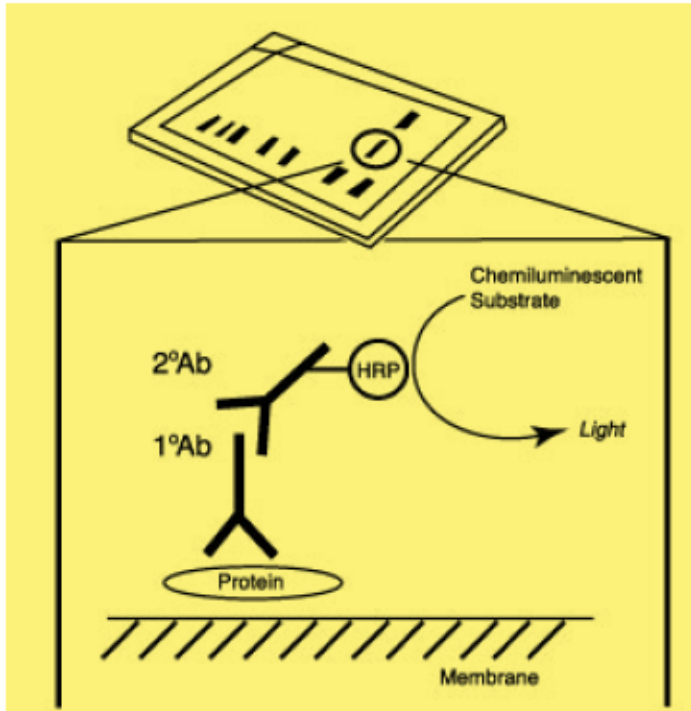
- 2x plastic containers (bench)
- flat forceps (bench)
- scalpel (bench)
- Ponceau S stain (bench, in the dark)
- 0.1M NaOH (bench)
- 1XTBST (bench)
- 1 clear plastic sheet (bench)
- Blocking Solution
- primary antibody (storage conditions dependent on antibody)
- 50ml conical tube

36. Press stop on the power supply, turn off the stir plate and return the apparatus to the bench.
37. On paper towel on the bench, open the sandwich (black side down) and cut the membrane to size using the scalpel, following the outline of the gel below it. If the transfer was successful, you will see the ladder on the membrane. Cut the nick in the corner again and flip the membrane over so that the nick is now on your left and place in a container. To keep track of which side is which, this nick should always be on your left, the side with your ladder. Repeat with the second sandwich.
38. Rinse the membranes with DW quickly then discard and add Ponceau S to the container (enough to cover the membrane). Place on belly dancer at low speed for 5 minutes.
39. During this staining, wash the transfer apparatus as you did the running apparatus. Allow to dry on the drying rack.

40. Discard the Ponceau stain down the sink and rinse the membranes with DW until all residual background red is gone, and only red bands remain. Scan this image on the computer.
 - a. Open Canoscan
 - b. Ethanol the scanner surface
 - c. Lay membranes down on the surface, ensuring no bubbles
 - d. Lay a clear plastic sheet over the membranes and close the scanner
 - e. Select "Scan1", source=platen, save to your file, click "ok"
 - f. After the scan completes, the image is saved automatically. Check to make sure the picture is clear before destaining the membrane
 - g. Ethanol the scanner surface again.
41. Destain the membranes by adding 0.1M NaOH to the container with agitation. It should destain within minutes.
42. Discard and rinse with DW, then wash the membrane for five minutes on the belly dancer at medium speed in 1XTBST (recipe in Buffers section, this can be made in advance and stored at room temperature)
43. During this wash step, prepare your blocking solution. Make sure to check the antibody information sheet of the antibody you will use to choose the appropriate blocking solution. Typically, 5% milk is sufficient, but BSA is also sometimes used. Skim milk is in a bag in the weigh room and BSA is kept at 4°C. Make this fresh daily in 1XTBST. Typically, 25ml is used per membrane.
44. Once the wash step is complete, discard the 1XTBST and add blocking solution to the container. Place on the belly dancer at room temperature on a low speed for 1 hour.

**Tip: If after you complete your western it comes out with nonspecific antibody binding, you can increase your blocking percentage to 8% to attempt to eliminate that.*
45. Just before the blocking step is complete, make up the primary antibody in a 50ml tube. The antibody information sheet should suggest a starting concentration for the antibody, as well as what to make it in. Typically, 5ml of antibody solution (5% milk or BSA made in 1XTBST) is made per membrane.
46. Once the blocking step is complete, if you are probing for multiple targets that run far enough apart on the gel, you can cut your membrane into two pieces and probe two at once. If you do this, you will cut using the scalpel, and use the smaller sectioned container. Each half of the gel should fit perfectly into the sections, allowing for the use of 2.5ml of antibody solution. If you do not cut the membrane, move the membrane to the

smaller coloured containers that fit the whole membrane perfectly. This container requires 5ml of antibody solution. Place the container of choice on the rocker in the fridge (4°C) at a low speed. Leave overnight.



The information sheet that comes with your antibody has suggested blocking percentages as well as antibody concentrations. It also lists species reactivity, meaning which animal species they can detect. Ensure the antibody you choose is specific for the species of your sample. Some primary antibodies are specific to multiple animals. Your secondary antibody is made to target your primary antibody based on the animal that your primary antibody was made in. So, if your primary antibody is a goat anti-rat IL-6, it is detecting rat

IL-6 in your sample, and was made in a goat. This means your secondary antibody must be anti-goat. Do not use a secondary antibody that is specific to your sample species. This will cause unspecific binding. So, if your sample comes from a rat, your secondary should not be anti-rat, and therefore your primary cannot be made in a rat.

Day 3 - Secondary antibody

What you need:

- 2x plastic containers
- 1XTBST (bench)
- Blocking solution (made fresh daily)
- secondary antibody (storage conditions dependent on antibody)
- 15mL tubes (bench)

47. Remove membranes from the fridge and place in plastic containers. Add 1XTBST to cover and wash the membranes for a total time of 25 mins (medium speed on the belly dancer), changing the buffer every 5 minutes (discard down drain).
48. During the last wash, prepare the secondary antibody as per the antibody information sheet. Typically, 25ml of solution (usually in 5% milk made in 1XTBST) is used per membrane.

49. Following washes, discard the 1XTBST and add the secondary antibody solution. Place on belly dancer for 1 hour at room temperature at a low speed.
50. Discard the secondary antibody solution and perform wash steps as per step 47.
51. During the final wash steps, prepare your detection solution (If using enhanced chemiluminescence, continue as below) and set up the computer

Enhanced Chemiluminescence (ECL)

What you need:

- 1XTBST (bench)
- 1.0M Tris pH 8.5 (4°C)
- DW
- 30% H₂O₂ (4°C)
- Coumeric acid - light sensitive (-20°C)
- Luminol - light sensitive (-20°C)
- 1x clear plastic sheet (bench)
- 2x 50mL tubes, one wrapped in tinfoil (bench, tinfoil in autoclave room)
- plastic wrap, taped flat to the bench
- kimwipes
- 1ml pipette and tips
- flat forceps for membrane handling

*Take out coumeric acid and luminol, wrap in tinfoil and thaw on bench

Label two 50ml tubes as "Solution 1" and "Solution 2". Add components listed below and keep solution 2 covered with tinfoil.

Solution 1		Solution 2	
Component	Volume	Component	Volume
1.0M Tris pH 8.5	2mL	1.0M Tris pH 8.5	2mL
30% H ₂ O ₂	12uL	90mM Coumeric acid	88uL
Distilled Water	8mL	250mM Luminol	200uL
		Distilled Water	8mL

Computer Set-Up

- a. Turn on the ChemiDoc imager (2 things to turn on: black box beside the computer first, then big beige imager)
 - b. Open "Quantity One" on the computer, press "EPI White" on the imager
 - c. In the program, "File" > "ChemiDox XRS" > "Select" > "Custom" > "Western MWM"
 - d. Change the filter on the imager to the middle position (black stick on top)
52. After the final wash, discard the wash buffer. Using the flat forceps, move the membranes to the plastic wrap.
53. Pour ECL solutions together into one 50ml tube and mix by inverting. Pipette the mixed ECL directly onto the membranes, being sure to cover every part of it. Continue for one minute.
54. Dab excess ECL solution from membranes onto kimwipe by touching the corner of the membrane to the kimwipes, handling with the flat forceps. Place membranes onto clear plastic sheet and move to imager
55. Open drawer on imager to place membranes on sheet inside. In program, click "Live Focus" > "Freeze" (once it is in the appropriate position; it can be focused using the buttons on the imager) > "Auto Expose" > "Save". You now have an image of your ladder saved which is used to determine band size.
56. To detect your chemiluminescence, "File" > "ChemiDox XRS" > "Select" > "Custom" > "Sean Bryan Western", turn off the "Epi White" on the imager, change the filter to the first position (a O), click "Live Acquire", and fill in as 300 second exposure with photos taken every 60 seconds. Click "Save" and it will run. This time can be altered based on your target protein and how easily it can be imaged. You will have an idea of how well this timing is working after the first minute when the first picture pops up.
57. Once all images have been taken, the membrane can be discarded or stored at 4°C in 1XTBS (1XTBST without the Tween 20) until a decision is made. The membrane can be stripped and re-probed for another target if necessary.

Buffers and Reagents

Tris 0.5M pH 6.8

Tris Base -12.1g
dH₂O - 200mL

Directions - Add Tris base to ~150mL of dH₂O, stir to dissolve completely. Adjust pH to 6.8 using concentrated HCl and adjust final volume to 200mL. Check pH and store in 4°C.

Tris 1.0M pH 8.5

Tris Base -24.23g
dH₂O - 200mL

Directions - Add Tris base to ~180mL of dH₂O, stir to dissolve completely. Adjust pH to 8.5 using concentrated HCl and adjust final volume to 200mL. Check pH and store in 4°C.

Tris 1.5M pH 8.8

Tris HCl-7.38g
Tris Base – 30.78g
dH₂O - 200mL
Adjust pH to 8.8 if necessary using concentrated HCl. Store in 4°C.

10% SDS

Sodium dodecylsulfate (SDS) - 10g
dH₂O - 100mL

4x Reducing Loading Buffer

0.5M Tris pH 6.8 - 10mL
dH₂O – 4.06mL
SDS – 2g
Bromophenol blue – 5mg
Glycerol - 10mL *add last!
-make 1ml aliquots and store at -20°C. Before use, add 110ul β-mercaptoethanol to 1ml.

1x Running Buffer

Glycine – 14.4g
Tris Base - 3g
SDS – 1g
Make up to 1L with dH₂O

1x Transfer Buffer

Glycine – 2.93g
Tris Base – 5.82g

dH₂O – 700ml
70% Methanol - 200ml
Make up to 1L with dH₂O, store at 4°C

10x TBS

Tris Base – 5.56g
Tris HCl – 24.24g
dH₂O – 800mL
-check pH, adjust with 12N HCl to pH 7.6
NaCl - 87.6g
Make up to 1L with dH₂O

1x TBST

dH₂O – 900mL
10xTBS – 100mL
Tween 20 – 1mL

Ponceau Stain

dH₂O - 475mL
Acetic acid - 25mL
Ponceau - 0.5g

*Note - light sensitive

Luminol

Luminol - 0.44g
DMSO - 10mL

Directions - Dissolve luminol in DMSO by vortexing. Pipette 250ul into Eppendorf tubes and store in -20°C. *Note - light sensitive

Coumeric Acid

Para-coumeric acid - 0.15g
DMSO - 10mL

Directions - Dissolve coumeric acid in DMSO by vortexing. Pipette 100ul into Eppendorf tubes and store in -20°C. *Note - light sensitive

8.3 Dual-Luciferase Assay SOP: LEES Lab

To be used on transfected cells (can be confluent myoblasts or differentiated myotubules)

Passive Lysis:

Complete once tubes are established, elongated, and about 70%. Take at least 4 pictures of plate before lysis > choose fields of view randomly

1. Catalogue media codes in "Media Sample Codes for ELISA" on Dropbox
2. Label 1.5ml Eppendorf tubes appropriately
3. Collect 300ul of media from each well and put into the labelled 1.5ml eppendorf tubes. Store in freezer boxes at -80°C for potential future ELISAs
4. Aspirate remaining media from each well and wash wells twice with DPBS, 2ml each well.
5. Add 500ul (for 6-well plate) of 1x passive lysis buffer (kept in -20°C at 5x) to each well and agitate on belly dancer (at 6) for 15 minutes.
 - a) 0.5ml/well (add one extra well to the math for pipetting error) = total volume needed (x). $x/5$ = how much passive lysis buffer needed, and make the rest of the volume up with DDW.
6. Parafilm the plates and place in -80°C chest freezer until luciferase assay can be done.

Preparing the plate:

7. Determine the samples you are going to run, and prepare a plate map in your lab book.
8. Determine the amount of reagent that is needed to run the assay (# of wells * 0.05 + 1ml). Take appropriate amount of LAR II and Stop & Glo buffer out of the -80°C chest freezer (middle section) and thaw in the dark at room temperature.
9. Obtain a white, half-well plate from the Lees bench shelf (top shelf by windows).
10. Immediately seal the plate with sealing tape for 96-well plates (found in first, top drawer from windows).
11. From your prepared plate map and using a scalpel, cut out the wells that will be used for samples (but remember to keep the tape available, so that it can be covered when you're completed).
12. Dispense 20ul of lysate (without trituration) into the appropriate wells (use a full box of pipette tips so that keeping your spot on the plate loading is easier – where you take a tip from is where you load on the plate).
13. Dispense the contents of the thawed reagents/buffers into 2 glass test tubes (1 for LAR II and 1 for Stop & Glo buffer).

14. Add Stop & Glo substrate to the Stop & Glo buffer (substrate is at 50x concentration so: $(\text{reagent volume} \times 1x) / 50x = \text{substrate volume needed}$).

Running the plate: FluoStar Optima set-up and use

15. Turn on the FluoStar Optima and the computer. When the computer welcome screen comes up, click on "Manager" and insert password (if you don't know it, ask Dr. Lees or Pam).
16. Since the luciferase assay is a luminescence assay, it is important to ensure the proper optics are in place inside the machine. Open the lid of the FluoStar Optima by pushing the silver handle towards the wall. For a luminescent assay, there should be only a blue excitation optic fiber coming from the middle of the machine (Note: everything is colour coded. If in doubt, ask Pam or Dr. Lees).
17. Open the FluoStar program on the desktop (top icon). When screen pops up, press "enter".
18. Within the program:
 - a) Setup tab > Reader Configuration > Luminescence > "Ok"
 - b) Test Protocols icon > double click "Dual Luciferase" then open the "Layout" tab and fill this in. Next open the "Concentrations" tab and set all concentrations to 50 > "Ok"
19. The luciferase assay uses the injectors, which need to be primed with the appropriate reagents. Click on the Erlenmeyer flask icon (prime) to begin priming the injectors.
20. Place the appropriate plastic tube of the injectors (#1 for LAR II and #2 for S&G) in the appropriate glass test tube. Place the injector at the mouth of each appropriate glass test tube as well (this is so the reagent is recycled back into the tube, so that no reagent is wasted).
21. Prime both injectors at least 2 times each. A warning will come up each time, just ensuring that you are not letting the primed product spill anywhere in the machine.
22. Once the injectors are primed, place them into the center of the inside of the machine (the black ring at the bottom has 2 holes for the injectors). Ensure they are completely inserted, but be very gentle with them.
23. Click the icon with the black bar and red arrow pointing outwards (to open the plate reader).
24. Close the plate reader with the button to the right of the "open" icon (black bar with red arrow pointing inwards).
25. While closing the optics lid (black cover with silver handle), ensure that the injector tubes (plastic ends without the injector probes) are

submerged in the glass test tubes to the very bottom. Slowly and gently close the lid.

26. Click the green light icon to begin reading. A window will pop up; select "Dual Luciferase" and then click "Start Test Run".
27. Once the run is complete, the tubes and injectors must be cleaned:
 - a) Move injectors to an empty beaker for waste, but leave plastic tubes in the glass test tubes
 - b) Open the priming window (Erlenmeyer flask icon) and select "Backflush", then prime each injector once (1ml)
 - c) Put the plastic tubes into a 15ml tube filled with 70% ethanol, select "Prime", and prime each injector (still in waste beaker) once with volume set to 4.5ml, then remove tubes from liquid, and prime once with volume set to 1ml
 - d) Repeat above but with plastic tubes in 15ml tube filled with DDW
 - e) Move plastic tubes into waste beaker with injectors and select "Backflush" again; prime each with volume set to 1ml
 - f) Move injectors back to original place, exit program and turn off machine.

8.4 Treatment Preparations and Calculations

8.4.1 Ascorbic Acid (AA) Preparation

First prepare 100 μ M AA:

Add 0.198g AA to 1 ml cell culture water (HyPure Cell Culture Grade Water – Thermo Scientific, cat # SH30529.02) for a 1 M stock concentration. Then sterile filter using a 0.28 μ m pore sterile filter and store at 4°C.

Next dilute 1M to 1mM:

$(10\text{ml cell culture water})(1\text{mM AA})/(1\text{M AA}) = 0.01 \text{ ml or } 10\mu\text{l of } 1\text{M AA into } 10\text{ml of cell culture water.}$

Finally calculate amount required to treat cells based on well size and volume of media:

E.g. if using a 6-well with 1.5ml media with a desired final concentration of 100 μ M (0.1mM) AA:

$(1.5\text{ml media})(0.1\text{mM})/1\text{mM} = 0.15\text{ml}$ or 150 μ l of AA into each well containing 1.5ml of media.

8.4.2 BMP-2 Preparation

Thaw a 10-15 μ l aliquot of 10 μ g/ml (10,000ng/ml) BMP-2 (Recombinant Human Bone Morphogenetic Protein-2 –Cedarlane, Burlington ON, cat # CLCYT261) from -80°C. Want to treat cells with a final concentration of 10ng/ml BMP-2.

Prepare a media + BMP-2 solution:

$(10\text{ng/ml BMP-2})(5\text{ml media})/10,000\text{ng/ml BMP-2 stock} = 5\mu\text{l}$ of 10,000ng/ml BMP-2 stock into 5ml of GM.

After preparing 10ng/ml BMP-2 solution change the wells on the plate with this media (*note if more than 5ml media is needed adjust accordingly).

8.4.3 Dual-Luciferase Reporter Assay Transfection Reagent Preparation

Before transfection can occur, the transfection reagent and plasmids must be prepared:

e.g. for CKM-luc: $((\text{number of wells} + 1 \text{ extra})(0.95 \mu\text{g CKM-luc})/\text{concentration of CKM-luc plasmid in } \mu\text{g/ml}) = \text{amount of MCK plasmid needed}$.

Calculate the same way for the amount of pRL-CMV *Renilla* needed however rather than 0.95 μ g use 0.05 μ g. Determine the volume of transfection solution required by multiplying the number of wells (including 1 extra) by 50 μ l. From this value, subtract the volumes of plasmids calculated from above to determine how

much 150 mM NaCl (Sodium Chloride – Fisher Scientific, Fair Lawn, NJ, cat # BP358-1) is required. Add the appropriate amount of NaCl to the same tube containing the plasmid and label Tube#1. For Tube#2, determine how much PEI and 150 mM needed to achieve a 3:1 ratio of PEI:DNA

e.g. 3 μ l of 10 mM PEI x number of wells = amount of 10 mM PEI needed for a (3:1 ratio of PEI:DNA).

Then,

47 μ l of 150 mM NaCl x number of wells = amount of 150 mM NaCl needed to keep 3:1 ratio of PEI:DNA.

8.5. BrdU “Part 2” and Flow Cytometry Preparation SOP: Lees Lab

Remove fixed samples from 4°C (see 3.3.4.1 for BrdU pulsing and fixing specifications). Transfer samples (after mixing) to pre-coated 15ml tubes. Tubes are pre-coated with 2-3ml of 1% BSA/DPBS solution for approximately 20 minutes. Centrifuge 15ml tubes @200xg for 15 minutes at RT. Decant BrdU from 15ml tubes using a pipet. Add 667 μ l of double-distilled water (DDW) to each tube, vortex, then add 337 μ l of 6M HCl to each tube. Vortex. Incubate 15ml tubes at RT for 30 minutes. After 30 minutes add 2ml of 0.1M Borate Buffer (Sodium Borate Decahydrate: Bioshop, ACS Reagent Grade, cat# SOB900.500) to each tube. Vortex, then centrifuge for 15 minutes @200xg. During this time, pre-coat 1.5ml Eppendorf tubes with ~1ml of 1% BSA/DPBS solution as above. After 15 minutes decant supernatant and add 1ml of 0.1M Borate Buffer to each tube. Vortex, then transfer mixture to pre-coated 1.5ml Eppendorf tubes. Centrifuge at 200xg for 15 minutes. Decant supernatant and add 1ml of DPBS to 1.5ml tubes

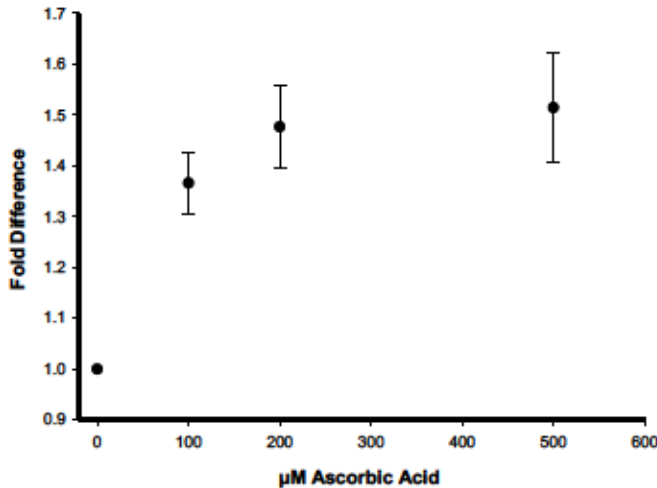
and vortex. Centrifuge 200xg for 15 minutes. During this 15 minutes prepare anti-BrdU (Fluorescein-anti-BrdU: Phoenix-Flow Systems, Sang Diego, CA, cat # ABFM-18). For anti-BrdU: 50µl of 0.1% BSA/DPBS is required per sample + 1 extra. Then 5µl per 100µl 0.1% BSA/DPBS of anti-BrdU is added (protect from light). After 15 minute centrifuge, decant supernatant and add 50µl of anti-BrdU solution to each tube, re-suspend by trituration. Incubate at RT in the dark for 30 minutes (turn on flow cytometer at this time). After 30 minutes add 400µl of DPBS to each tube and, after mixing, transfer entire volume to flow tubes. Run flow cytometry on samples as per section 3.3.1.4.

8.6 Plate Specifications Table for MPCs

Plate Type	Matrigel Volume	Media Volume	Trypsin Volume
12-well	0.5 ml	1.0 ml	0.5 ml
6-well	1.0 ml	1.5 ml	1.0 ml
60 mm dish	2.0 ml	2.5 ml	0.5 ml
T25 flask	2.5 ml	5.0 ml	2.0 ml
150 mm dish	N/A	20 ml	8.0 ml

*Media volumes are low for 60 mm and 150 mm culture dishes because proliferation experiments are carried out in 6% O₂ and a lower volume of media is required for appropriate oxygen diffusion into the cells.

8.7 Supplementary Figures and Data



Appendix Figure 1. Dose response of superoxide production from ascorbic acid utilizing the Nitroblue Tetrazolium (NBT) assay.

These results are obtained from unpublished preliminary data from the laboratory of Dr. S.J. Lees. An NBT assay was conducted to determine at which dose ascorbic acid optimally functioned as a pro-oxidant to increase production of superoxide (a type of reactive oxygen species or ROS). It was determined that a dose of 200µM ascorbic acid, superoxide production was most optimal therefore this dose was chosen for this study.

8.8 List of Abbreviations

- AA – Ascorbic Acid
- ALP – Alkaline Phosphatase
- ARS – Alizarin Red Stain
- BMP – Bone Morphogenetic Protein
- BrdU – Bromodeoxyuridine
- CAT – Catalase
- CPC – Cetylpyridinium Chloride
- DDW – Double-Distilled Water
- DM – Differentiation Media
- DMEM - Dulbecco's Modified Eagle Medium
- DMSO - Dimethyl sulfoxide
- DPBS – Dulbecco's Phosphate-Buffered Saline
- DW – Distilled Water
- FBS – Fetal Bovine Serum
- Geo Mean – Geometric Mean
- GM – Growth Media
- H₂O₂ – Hydrogen Peroxide
- HG – High Glucose
- HO – Heterotopic Ossification

MAD – Mesenchymal Alternative Differentiation
MG – Matrigel
MOP – Myositis Ossificans Progressiva
MOT – Myositis Ossificans Traumatica
MPC – Muscle Precursor Cell
MSC – Mesenchymal Stem Cell
NBT – Nitroblue Tetrazolium
NSAID – Non-Steroidal Anti-Inflammatory Drugs
PEG-Cat– Polyethylene Glycol-Catalase
PEI – Poly(ethyleneimine)
PGE-2 – Prostaglandin E2
pNPP – p-Nitrophenyl phosphate
PPM – Pre-Plate Media
RDA - Recommended Dietary Allowance
ROS- Reactive Oxygen Species
RT – Room Temperature
SEM – standard error of mean
SOD – Superoxide Dismutase
TEMPOL – 4-Hydroxy-TEMPO
WR – Working Reagent