

ISOLATION AND CULTURE OF MYOFIBER-DERIVED CELLS FROM THE
EXTENSOR DIGITORUM LONGUS MUSCLE

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ABSTRACT

Isolation and Culture of Satellite Cells from the Extensor Digitorum Longus

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Peripheral arterial occlusive disease (PAOD) involves distal artery occlusion by atherosclerotic plaques, which restricts blood flow and leads to ischemia in downstream tissues. Increased blood flow through preexisting collateral vessels leads to increased shear stress that triggers an outward remodeling of the vessel called arteriogenesis. In some cases this natural compensatory mechanism is able to sufficiently restore blood flow following arterial occlusion. However, for many individuals this process is insufficient to relieve peripheral ischemia, and patients experience intermittent claudication, or limb pain with locomotion or exercise. Without treatment, reduced blood flow can lead to tissue necrosis and potentially amputation. The efficacy of medication, such as drugs to lower cholesterol, is limited while surgical intervention is only available to a limited patient population. Supervised exercise therapy can improve important patient outcomes such as pain-free walking time and distance. Unfortunately, many patients fail to adhere to regimented exercise programs, limiting its functional efficacy. A potential alternative treatment approach is to stimulate or enhance arteriogenesis with cell therapy to recapitulate the beneficial effects of exercise therapy. Following exercise, resident muscle stem cells known as Satellite Cells (SCs) activate, repairing damaged muscle fibers and playing an important role in local angiogenesis. They and their progeny myoblasts, secrete a wide range of factors known to be involved in arteriogenesis and the recruitment of other cells involved in remodeling. As such, these cells are ideal candidates for transplantation. Before preliminary evaluation of these myogenic cells can be performed, a reliable source of these cells is required. Towards this goal, protocols were developed to obtain cells in sufficient numbers for transplantation. Whole Extensor Digitorum Longus (EDL) muscles were excised from mouse hindlimbs. Myofibers were isolated by collagenase digestion and trituration, and placed in specialized culture conditions to promote the adherence and selective growth of myogenic cells. In these conditions, SCs activate, migrate from myofibers, and proliferate. With increasing time in culture, especially following myofiber hypercontraction, SCs increasingly differentiate, limiting expansion and the availability of cells for transplantation. Future research will focus on improving culture conditions to inhibit differentiation and maintain sustained growth to improve the availability and in vivo behavior of isolated cells. Once sufficient cell numbers are obtained they will be transplanted into a mouse model of chronic peripheral ischemia to investigate their impact on collateral remodeling.

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“When you want to give up, try one more time.”

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Chapter 1: Introduction

1.1 Relevance: Peripheral Arterial Occlusive Disease

Peripheral Arterial Occlusive Disease (PAOD) is an ischemic disease characterized by the narrowing, or stenosis, of distal arteries, primarily in the lower extremities. Most commonly stenosis results from atherosclerotic plaques within arterial walls. With atherosclerotic progression, vessels can become increasingly occluded, leading to ischemia in downstream tissues. Alternatively, thromboembolism can lead to a rapid onset of ischemic conditions¹⁻³. In the United States, over 8 million individuals have PAOD, with older individuals being disproportionately affected^{4,5}. Patients with PAOD commonly present with temporary pain during activities such as walking or exercise, which is

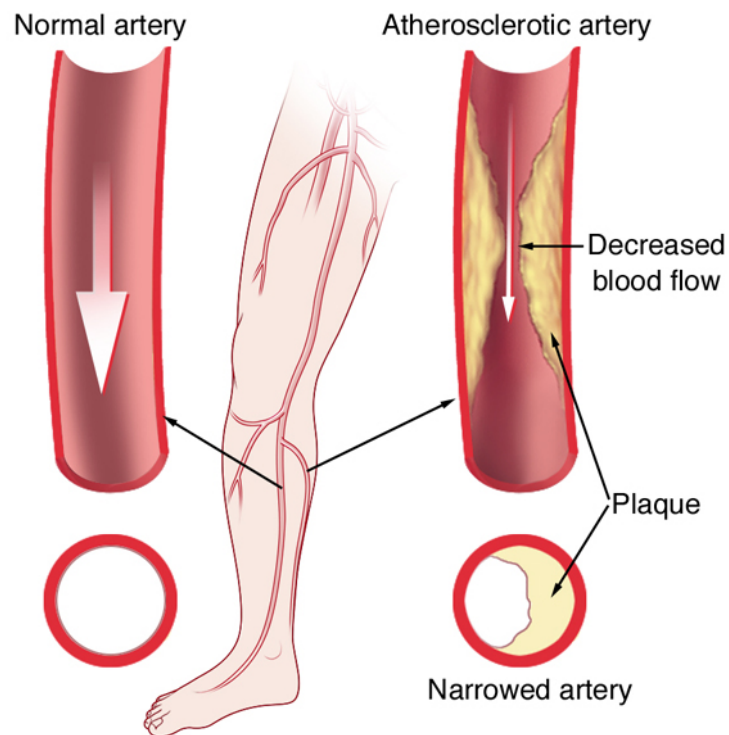


Figure 1. Peripheral Artery Occlusive Disease. In atherosclerosis, plaques develop within the walls of conduit arteries reducing blood flow and causing ischemia downstream tissues⁶.

termed intermittent claudication ². If left untreated, blood flow can be further reduced until insufficient oxygen and nutrients are delivered even at rest, which is described as critical limb ischemia (CLI) ⁷. Within one year, 30% of individuals undergo amputation and another 25% of individuals die from coronary heart disease or stroke, which commonly present with PAOD.⁸. Although only a small percentage of patients reach this advanced stage of the disease, up to 50% of patients with PAOD experience leg symptoms that reduce mobility, leading to reduced reported quality of life, and increased rates of depression⁹

First line treatments focus on the reduction risk factors for atherosclerosis, including cessation of smoking and management of blood pressure and cholesterol¹⁰. While these treatments are relatively low risk, they have a limited ability to address the underlying problem of reduced peripheral circulation. For example, cholesterol lowering medications may reduce the likelihood of plaque growth without substantially reducing plaque size¹¹. Alternatively, surgical or endovascular procedures including bypass grafts and angioplasty, respectively, can be utilized to restore blood flow ^{2,10}. Unfortunately, patients only become eligible for these procedures after failure of first line treatments or in the event of severe claudication leading to CLI ¹². Another treatment strategy, often prescribed as a first line treatment, is the adoption of a regimented exercise program. Treatment commonly consists of a minimum three month program with increasing walking times or distances where patients are only allowed to take breaks with the advent of moderate to severe leg pain ⁷. Adoption of such a program significantly increases both pain-free walking time and distance, but its efficacy is limited by poor adherence to exercise ^{10,13}. Compliance in clinical trials is highly variable with one study reporting

compliance as low as 49%¹. Currently, PAD lacks treatment options that both treat the underlying disease and are available and reasonable for most patients to complete.

1.2 Potential Treatment: Collateral Arteriogenesis

A potential alternative approach for treatment of PAOD is to enhance the growth of natural bypass vessels within the peripheral circulation¹⁴. Some individual arterial trees are connected via small arteriole anastomoses, i.e. collaterals¹⁵. In the distal ischemic hindlimb, collaterals function as the primary site of flow resistance¹⁶. Through a process known as collateral arteriogenesis, these vessels have the ability remodel into high conductance, low resistance arterial vessels, a process which can be enhanced with exercise¹⁷⁻¹⁹. When an arterial vessel is occluded, an increased pressure gradient is established across collaterals upstream of the occlusion²⁰. As a result, blood flow is elevated through these collaterals, causing them to act as a natural bypass routes between adjacent arterial trees²⁰. Simultaneously, increased blood flow increases shear stress, initiating collateral arteriogenesis^{20,21}. Endothelial cells (ECs) recruit monocytes which extravasate and differentiate into macrophages that orchestrate degradation of the extracellular matrix (ECM)^{22,23}. Additional cytokines released by ECs promote proliferation of both themselves and smooth muscle cells before new ECM is synthesized by local cells^{22,23}. When completed, remodeled collaterals have the potential to restore sufficient perfusion to ischemic tissue downstream of an occlusion.

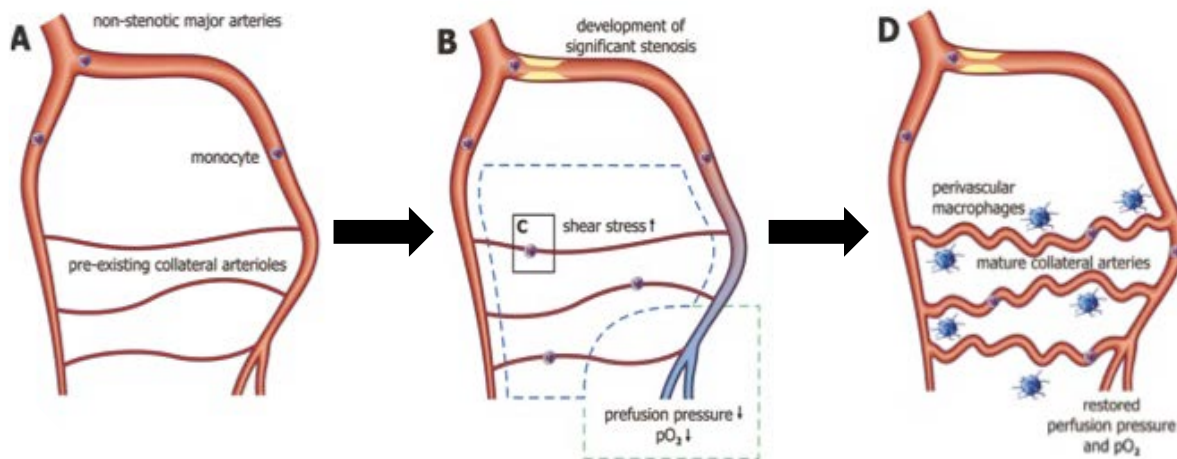


Figure 2. Arteriogenesis. Development of atherosclerotic plaques reduce blood flow to downstream tissues while simultaneously increasing shear stress in pre-existing collateral arterioles. This high shear condition activates endothelial cells, leading to the outward remodeling of these bypass vessel and restoring perfusion to affected tissues.²⁴

Unfortunately, this endogenous mechanism of collateral arteriogenesis fails to sufficiently restore blood flow for many individuals²⁵. Variation in both the preexisting collateral network and magnitude of the arteriogenic response varies significantly in animal models and is suspected to be similarly present with the human population^{15,25}.

1.3 Cell Therapy: Satellite Cell Transplantation

To date, attempts to treat PAOD by augmenting collateral arteriogenesis have proven unsuccessful. Exercise treatment is efficacious, but poor compliance prevents widespread implementation ref. Delivery of individual arteriogenic factors (VEGF, bFGF, etc.) using gene therapy or recombinant proteins have appeared promising in preclinical animal studies and small uncontrolled clinical trials, but have proven unsuccessful in large, controlled clinical trials^{26,27}. Specifically, these therapies failed to improve pain-free walking distance or ankle brachial index^{14,28}. Alternatively, transplantation of cells involved in collateral remodeling are being investigated to recapitulate the benefits of exercise therapy⁹. Instead of delivering an individual growth factor, these cells can

potentially act as “cytokine factories”, producing a wide range of growth factors²⁹. For example, bone marrow derived cells (BMC) are being investigated due to their recruitment to collaterals and secretion of a wide variety of cytokines (MCP-1, FGF-2, TGF- β , uPA, and MMPs) involved in collateral remodeling²⁹. Another cell type integrally involved in exercise is the Satellite Cell (SC), the resident stem cell responsible for the repair and maintenance of skeletal muscle. In response to muscle damage or exercise, SCs activate and differentiate into highly proliferative muscle precursor cells (MPCs) also known as myoblasts. With further differentiation, myoblasts exit the cell cycle and fuse, incorporating into regenerating or repairing myofibers^{30,31}. SCs are preferentially located near capillaries within skeletal muscle and are implicated in signaling with endothelial cells to simultaneously promote myogenesis and angiogenesis³². Additionally, activated SCs and myoblasts secrete a variety of factors involved in collateral remodeling (MMPs, VEGF) and recruitment of monocytes (MCP-1, MDC, FKN)^{23,31}. Therefore, SCs and myoblasts are attractive transplantation candidates to recapitulate the beneficial effects of exercise to treat PAOD.

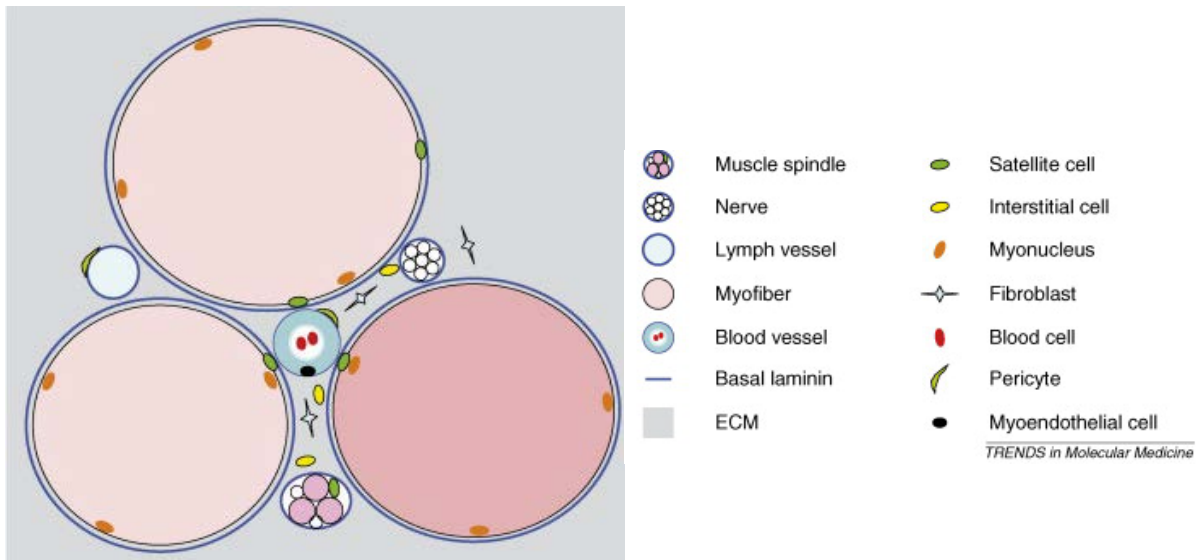


Figure 3. Satellite stem cell niche. Satellite cells are located on the periphery of myofibers between the basal lamina and sarcolemma in close proximity to the local microvasculature. Adapted from ³³

1.4 Project Objective

To evaluate the potential effects of SC transplantation on arteriogenesis, it is necessary to establish a reliable method to isolate and culture primary murine SCs. The objective of this project is to build off previous work conducted to isolate and culture SCs from the Extensor Digitorum Longus (EDL) hindlimb muscles of mice ¹⁵ and establish reliable protocols to:

1. Excise whole EDL muscles from adult mice
2. Isolate individual viable myofibers from explanted muscles
3. Optimize a primary culture system of live myofibers to allow sufficient expansion of SCs for transplantation

Chapter 2: Methods and Results

2.1 Animal Husbandry

Experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Cal Poly. Male and Female C57Bl/6 and ICR mice aged between 2-7 months were used for all experiments. Mice were maintained on a 12:12 hour light:dark cycle with ad libitum access to feed and water.

2.2 Extensor Digitorum Longus Excision

Mice were euthanized by cervical dislocation while anesthetized with 1-3% isoflurane gas with a flow rate of 0.8-1 l•min⁻¹. Mice were placed in a supine position on a Styrofoam working surface, all four limbs were pinned to the surface with XXG syringes, and both hindlimbs were thoroughly disinfected with 70% isopropanol. The skin was then removed from the knee to the medial aspect of the hindpaw. The distal tendon of the Tibialis Anterior (TA) and Extensor Digitorum Longus (EDL) were exposed and the medial aspect of the TA was freed from the tibia by cutting along its length. Next, the TA was resected and discarded to improve visualization before resecting the EDL. Special care was taken to ensure that the EDL was minimally damaged throughout the dissection process. Muscles were only manipulated via the proximal and distal tendons to avoid contact with the muscle body. To reduce the chance of stretching the muscle, manipulation under tension was avoided. While grasping the tendon, the muscle belly was allowed to sag, providing a margin of error before muscle damage would occur. Loss of fiber integrity at this stage, due to even minor stretching, leads to excessive fiber death and hypercontraction during subsequent digestion steps^{34,35}.

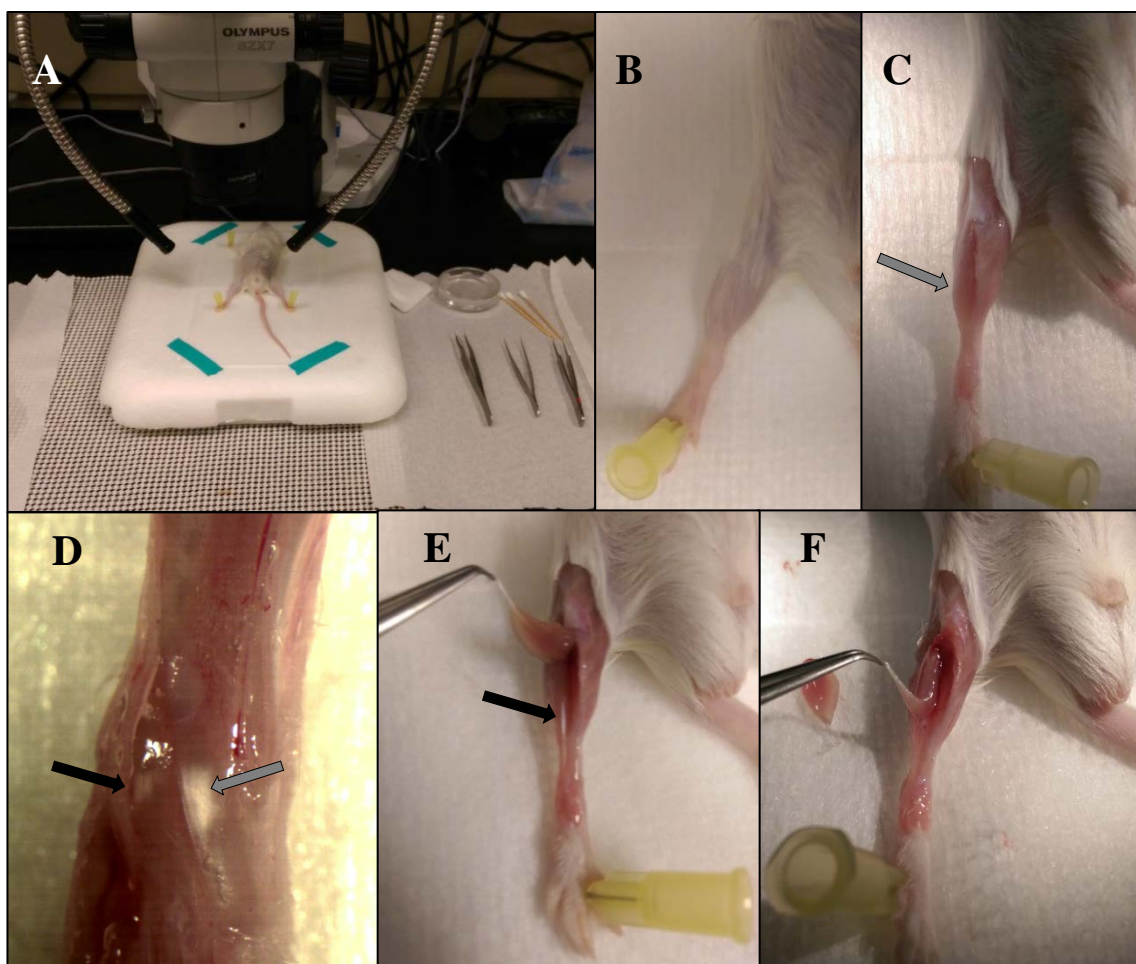


Figure 4. Extensor Digitorum Longus excision. (A) Experimental set-up used for the excision of EDL muscles. Removal of skin of the hindlimb (B) exposes the TA muscle (C, grey arrow) and distal tendons (D) of the TA (grey arrow) and EDL (black arrow). (E) Excision of the TA (E) exposes the EDL muscle (black arrow). Handling only the distal tendon, the EDL is gently excised from tendon to tendon (F)

2.3 Enzymatic Digestion

Excised muscles were immediately placed in a digestion solution (3mL, 2 mg·mL⁻¹ type 2 collagenase [Worthington CLS-2], 1% Penicillin-Streptomycin [Omega PS-20] in HAMS F10 [Fisher SH3002501] in a water bath at 37⁰C. Every ten minutes the solution was inverted to ensure even digestion. Muscles were incubated for 50-70 min depending upon muscle appearance; after 50 min, muscles were visually inspected every 5 minutes to avoid over-digestion of the muscle. When an EDL muscle has been properly digested, the muscle will shorten and widen as individual muscle fibers separate from each other and

structural connective tissue degrades. Distinct, shiny myofibers will begin to protrude from the muscle body. In the case muscles were overdigested, the muscle will appear fuzzy and milky colored as the surface fibers have died and hypercontracted.

2.4 Mechanical Myofiber Dissociation

All manipulations of muscles or myofibers were carried out using custom, beveled, glass pipettes coated with horse serum. Horse serum is critical to prevent adhesion of the myofibers to the pipette. Upon sufficient digestion, muscles were transferred to a 100 mm tissue culture dish filled with wash media (HAMS F10 supplemented with 10% FBS [Fisher SH3091003], 1% Pen-Strep) using the largest diameter pipette (4mm i.d.). Muscles were then washed with media and gently triturated to release individual fibers. This process was repeated with two progressively smaller pipettes (2, 0.75 mm i.d. respectively) until all live fibers were harvested. Live and dead/damaged fibers were distinguished based upon morphology. Live fibers were straight or wavy and appeared translucent or iridescent depending upon lighting conditions. With sufficient time in culture, fibers with a wavy appearance adopt a straight appearance. If a fiber was severely damaged, hypercontraction would occur at the site of injury, appearing as a widened, milky-colored section of fiber. Often, this leads fibers to have one or more sharp kinks along their length. In most cases, the area of hypercontraction would extend along the entire length, leading to death of the fiber. Under a stereo dissecting microscope, using a horse serum coated P100 pipette, live myofibers were transferred into a series of five tissue-culture dishes with wash media (20 mL dish 1-2, 10 mL dishes 3-5) to enrich for live fibers and reduce non-myogenic debris. Enriched fibers were plated

in a 60 mm tissue culture dish that was previously coated overnight with 1:1 ratio of collagen I (Fisher CB354249) and laminin (Life Technologies 23017015) and rinsed three times with wash media. The final plate was filled with 6 mL of wash media containing 2 ng/mL bFGF (Fisher CB40060), and incubated at 37°C with 5% CO₂.

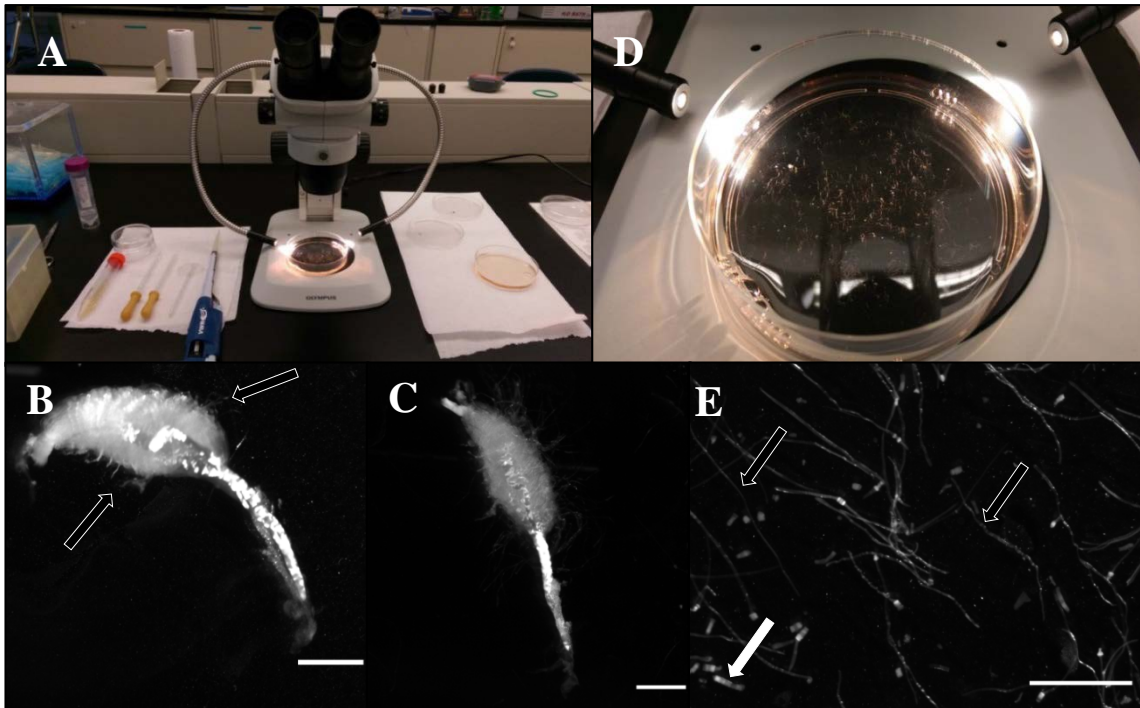


Figure 5. Isolation of live myofibers. (A) Experimental setup used to isolate individual live myofibers. (B) Complete enzymatic digestion with myofibers protruding from the muscle (black arrows). (C) Fibers are released from the muscle with mechanical dissociation resulting in (E) a mixed culture containing both live (black arrows) and dead (white arrows) fibers. Images were obtained using a stereo dissecting microscope. All scale bars are 500 μm .

2.5 Myofiber Culture

Myofiber cultures were assessed daily using a phase-contrast microscope. Typically, punitive Satellite Cells began migrating from fibers after two days in culture. Every three days, media was partially exchanged ($\sim X\%$) and supplemented with bFGF (2 ng $\cdot\text{mL}^{-1}$); full media exchange was avoided to minimize fiber loss. Cultures were maintained in the

initial dish until the majority of myofibers had died or the confluency reached 80%. For passaging, strong attachment of fibroblasts was exploited to enrich the culture for myogenic cells. Briefly, the media was aspirated, trypsin was added, and the culture was placed in the incubator. After one minute, cultures were observed to ensure selective liberation of myogenic cells (based on morphology) from the dish bottom. Trypsin (Invitrogen 25300-062) was neutralized with an equal volume of wash media and the cell suspension was centrifuged at 0.3 rcf for 10 minute. The resulting cell pellet was resuspended in wash media, and cells were plated onto coated dishes at approximately 20% confluency. Cultures were subsequently passaged upon 80% confluency or after 5 days³⁶. Myoblasts adopt a spherical or spindle-like morphology upon attaching to the culture dish becoming increasingly differentiated with time in culture (**figure 6 D**).

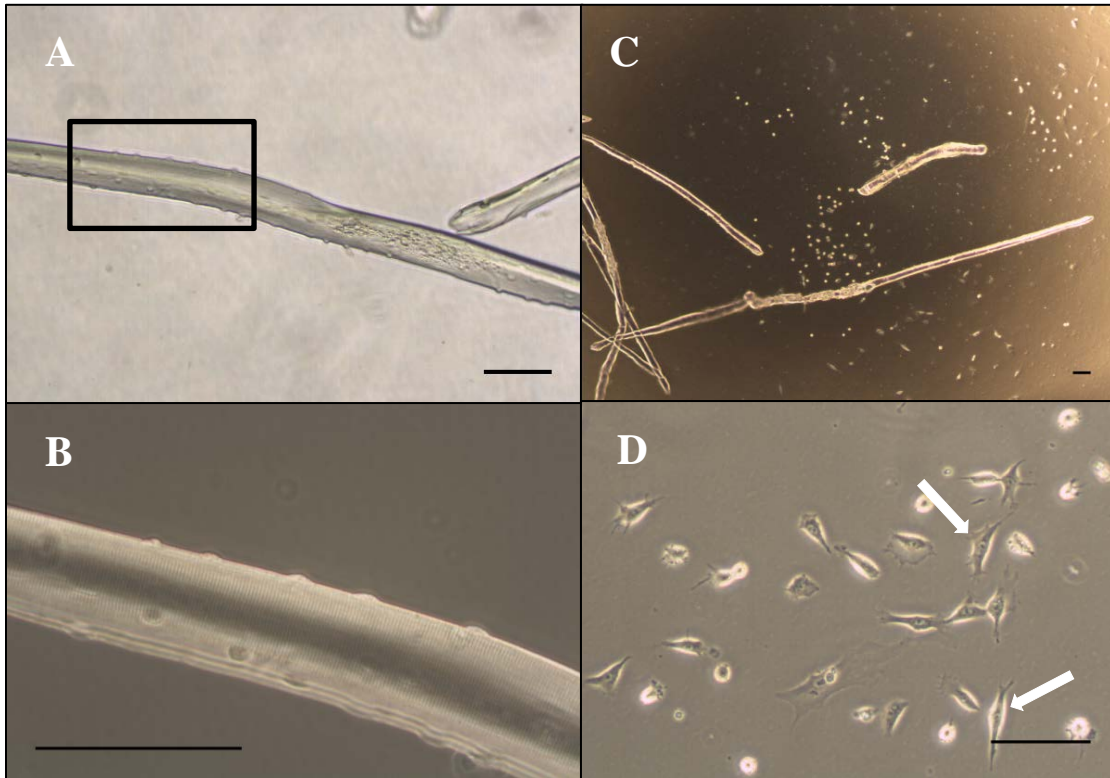


Figure 6. Culture of live myofibers. (A) Representative image of a live myofiber following isolation. (B) At higher magnifications cell nuclei and striations can be observed (C) punitive Satellite cells begin to migrate from fibers after 48 hours in culture. (D) With time in culture myogenic cells become increasingly differentiated appearing increasingly elongated (white arrows). Representative images were obtained using a phase contrast microscope. All scale bars are 50 μm .

Chapter 3: Discussion

3.1 Protocol Development – Extensor Digitorum Longus Excision

PAOD affects over 8 million Americans, commonly causing pain with movement or exercise that leads to restricted mobility and a lower quality of life. Progression of PAOD can lead to amputation and is associated with increased risk of mortality. Most widely available treatments fail to address underlying deficits in peripheral circulation, and treatments such as exercise therapy which improve collateral circulation have poor compliance. Cell therapy has been proposed as an alternative strategy to recreate the beneficial effects observed with exercise. Specifically, the objective of this project was to isolate and culture myogenic cells for subsequent evaluation of their impact on collateral arteriogenesis. As a first step, EDL muscles and their proximal and distal tendons were exposed and subsequently excised. The extent of connective tissue obscuring these tendons was highly dependent upon mouse age. In young mice both distal tendons could be easily visualized and accessed with minimal blunt dissection, whereas older mice required extensive blunt dissection to visualize distal tendons. To reduce the likelihood of unintentional muscle damage, young mice should be used whenever possible.

3.2 Protocol Development – Isolation of Individual Myofibers

The connective tissue of isolated whole muscles was degraded using a type II Collagenase solution, based on previous work²³. Muscles were incubated without agitation and periodically mixed to ensure even enzyme coating for uniform muscle digestion³⁵. Although gentle agitation increases SC activation during digestion of Flexor Digitorum Brevis muscles, this technique was not utilized³⁷. Access to the necessary equipment was intermittent and risked cooling the muscle significantly below body

temperature. Additionally, the culture conditions used are sufficient to activate SCs^{34,38}. Determination of the appropriate length of digestion proved to be a significant challenge. If fibers were digested for too short a time, connective tissue would be insufficiently degraded to allow for liberation of individual fibers from the muscle belly. When triturating or washing media over the muscle, superficial fibers would only separate if excessive force was used. Fibers liberated in this manner would commonly die due to damage inflicted from the process. Simultaneously, deep connective tissue would fail to be noticeably degraded. In contrast, excessive digestion would severely damage myofibers leading to a 'kinked' morphology. After a short time in culture these fibers die and develop a hypercontracted appearance³⁴. Fibers were most effectively isolated when muscles were digested in the collagenase solution until the tips of superficial fibers began to protrude from the muscle belly. Complicating matters, the time required to reach this level of digestion was highly dependent upon the muscles' physical characteristics. EDL muscles are commonly used to isolate live myofibers due to their thin shape and minimal connective tissue, both which aid enzymatic digestion. With increasing age, mouse EDL muscles increase in both collagen content and total volume of connective tissue³⁹. The EDL muscles of adult mice (5-7 months) are significantly larger than those of young mice (3 months) 15.4±3.2 mg versus 9.04±0.2 mg respectively^{40,41}. Using the described digestion conditions, fewer viable myofibers were harvested from older adult C57Bl/6 mice compared with younger mice. Even with longer digestion times larger muscles failed to digest uniformly. Superficial fibers degraded and died while deep connective tissue remained intact. A potential explanation was that the digestion used was too aggressive leading to excessive cellular damage. In future work, this problem may be

addressed through the use of type I Collagenase, combined with a longer digestion time⁴². Type I Collagenase has reduced activity of Clostripain, a proteinase that degrades arginine, making it more suitable for the digestion of delicate tissues such as liver or adrenal gland⁴³. In fact, some protocols involve the use of type I Collagenase coupled with a longer digestion to isolate EDL and the larger Tibialis Anterior (TA) muscle^{34,38}. Alternatively, the working concentration of type II Collagenase could be reduced to increase digestion uniformity. For either approach, it is important to account for significant lot-to-lot variation in enzyme activity. In fact Worthington Biochemical, the supplier of the type II Collagenase, only reports a minimum value of enzymatic activity and highly recommends sampling multiple lots for ideal digestion characteristics⁴⁴. As all these factors modify digestion time, it is important to closely monitor the muscle digestion process in every procedure.

3.3 Protocol Development – Mechanical Dissociation of Individual Myofibers

Upon complete digestion, muscles were transferred to a fresh tissue culture dish for mechanical dissociation of individual fibers. As with other steps, significant effort was made to minimize damage to live myofibers. Surfaces in contact with myofibers were coated horse serum to minimize adhesion. Media was gently washed over the muscles, isolating fibers from the muscle body using a wide bore glass pipette. Although some protocols caution against trituration of the muscle, the technique proved useful for separating more strongly attached fibers from the muscle belly³⁴. If used, it is critical that the mouth of the pipette is large enough to allow the muscle to freely enter the pipette without making contact with the pipette mouth. As fibers liberate from the muscle, smaller bore glass pipettes maintain trituration intensity. Failure of fibers to separate

indicates the muscle was not sufficiently digested or was severely damaged, which cannot be addressed by increasing the trituration force. Few fibers releasing, with minimal kinked or hypercontracted fibers, suggests insufficient digestion which can be rectified by further digesting the muscle in the collagenase solution. On the other hand, significant debris and hypercontraction suggest excessive fiber damage due to digestion or surgical error, which cannot be salvaged. If digestion and trituration are performed effectively, freed myofibers appear shiny and straight or wavy (a wave-like morphology should become straight after resting in culture overnight). Inevitably, the dissociation process releases a wide range of undesired cell types, including adipocytes and fibroblasts. To reduce contamination of the culture with these cells, live myofibers were identified and repeatedly transferred to fresh culture dishes. Initial use of three transfers proved insufficient, resulting in cultures with mixed populations of cells. Although unobservable, free-floating, non-myogenic cells are present in media after 3 washes⁴⁵. The addition of two wash steps proved sufficient to significantly reduce contaminating cells, producing predominantly myogenic cultures⁴⁵.

3.4 Protocol Development – Live Myofiber Culture

Isolated myofibers were immediately transferred to a culture system to selectively expand myogenic cells. To efficiently expand these cells, in vitro conditions must be designed to recapitulate as many aspects as is reasonable of the satellite cell niche. Together, the media and culture substrate must contain the nutrients, soluble factors, and physical cues necessary to support cell survival and proliferation. In this culture system, HAMS F10 nutrient mixture was chosen as the media base because it provides a selective growth advantage for myogenic cells⁴⁶. In contrast, in other nutrient mixtures such as DMEM,

myoblasts expand more quickly, but are quickly outcompeted by fibroblasts.⁴⁶ Although precautionary steps were taken to reduce non-myogenic cells, isolated populations of fibroblasts were still commonly observed, necessitating use of HAMs F10. However, once a highly myogenic population is established, DMEM could potentially be used to improve the expansion rate of the culture.

Base media was supplemented with fetal bovine serum (FBS), an essential media component commonly used to culture myoblasts, that provides a relatively high concentration of necessary nutrients and growth factors^{38,46,47}. Myofibers were initially cultured in media containing 10% FBS, as myofibers survive longer in a relatively low serum environment³⁸. Under these conditions SCs activate and migrate from fibers. Once fibers die in culture, the serum concentration was increased to 20% to encourage proliferation³⁸. Survival of myofibers was prioritized over increased serum concentrations due to an observed reduction of myoblast proliferation rates following myofiber death, even with increased serum concentrations. This observation may be explained by a loss of myofiber-derived growth factors (IGF, EGF, HGF) that encourage proliferation^{30,31}. In addition to FBS, the culture was supplemented with basic fibroblasts growth factor (bFGF or FGF2). Found in the basal lamina of skeletal muscle, bFGF is released upon muscle injury and has been implicated in the activation, proliferation, and reduced differentiation of SCs^{30,31,48}.

To facilitate attachment of SCs, culture dishes were coated with two skeletal muscle ECM proteins, collagen I and laminin. Collagen I is a major structural component of the ECM that is commonly-chosen culture substrate, and is deposited by myogenic cells in

vitro^{46,49}. Laminins are glycoproteins that act as linkage molecules between the basal lamina and sarcolemma transmitting both mechanical forces and chemical signals⁵⁰.

Interestingly, the enhanced proliferative effects of bFGF are dependent upon interactions with the ECM, increasing specifically with laminin as a culture substrate⁴⁸. Broadly, interactions of SCs with the ECM are implicated in regulating a wide range of satellite cell behavior³⁰.

Using the described culture conditions myofiber derived cells were consistently expanded. However, cells began to increasingly exhibit a wide range of morphologies and variable proliferative potentials with increasing time in culture, an effect that was accelerated with loss of live myofibers in culture. Some cells retained a compact circular phenotype characteristic of minimally differentiated cells, while others exhibited an elongated bipolar phenotype. With increasing differentiation, the proliferative ability of the cells in culture declined. Once a majority of the cells appeared differentiated, the culture failed to appreciably expand.

3.5 Future Work

Future work will focus on improving culture conditions to enable long-term expansion of myogenic cells. Current culture conditions allow for initial expansion followed by a progressive loss of proliferative potential. Commonly, only $1-2 \times 10^5$ cells are generated, limiting the number of transplantation experiments that can be completed with a single isolation of SCs. Additionally, current conditions result in progressive uncontrolled differentiation of myogenic cells. When considering cell transplantation, it is important to consider how in vitro differentiation will affect cellular behavior in vivo. Even three days in culture resulted in a tenfold reduction in the engraftment efficiency of FACS isolated

SCs in a dystrophic mouse model⁵¹. Similarly, in the context of peripheral ischemia, the function of transplanted myogenic cells may be reduced with SC differentiation. To address this concern, a culture system designed to limit differentiation should be investigated. One particularly interesting approach involves the use of a small molecule inhibitor of p38 MAPK signaling. p38 MAPK is part of a transcriptional regulatory network that influences SC self-renewal and differentiation⁵². FACs isolated Human SCs treated with this inhibitor suppressed expression of several differentiation factors while maintaining exponential growth for three weeks in culture⁵². When implanted, these cells demonstrated improved engraftment compared to freshly isolated or standard culture conditions⁵². Adaptation of this system to the described single fiber culture has the potential to prevent in vitro differentiation, allowing long term culture expansion and improved in vivo behavior of myogenic cells for future transplantation studies.

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Appendix

Muscle Fiber Preparation and Myofiber Isolation

Date: _____

- Type II Collagenase
- HAMS F10 Nutrient Mixture
- Pen-Strep
- Horse Serum (HS)
- Fetal Bovine Serum (FBS)
- Calf Skin Collagen Stock Solution
- 18 M Ω Water
- Glacial Acetic Acid
- 60 mm Tissue Culture (TC) Dish
- 100 mm Tissue Culture (TC) Dish
- 15 mL Conical Tubes
- 50 mL Conical Tubes
- 37⁰C Water Bath
- Rocking Platform
- Sterile PBS
- P1000 Micropipette
- P1000 Micropipette Tips
- Standard Pattern Forceps
- Curved Iris Scissors
- 5/45 Forceps
- Beveled Glass Pipettes
- Cotton swabs
- Cotton patches
- 500 mL, 0.22 μ m Vacuum Filter System
- Isopropyl Alcohol (IPA)

Muscle Fiber Wash Plates

1. ___ Defrost aliquot of HS
2. ___ In TC hood, Coat 5x 100 mm TC dishes in HS
 - a. Label TC dishes 1-5
 - b. Pipette 7 mL of HS into first TC dish
 - c. Transfer HS between dishes
 - d. Place remaining HS in 15 mL conical and place in 37⁰C water bath
 - e. Aspirate excess HS from TC dishes
3. ___ Place 20 mL of wash media into Dishes 1-2
4. ___ Place 10 mL of wash media into Dishes 3-5
5. ___ Warm plates in 37⁰C, 5% CO₂ incubator

Muscle Excision – Extensor Digitorum Longus (EDL)

1. ___ Euthanize mouse
2. ___ Use insulin syringe needles to secure fore and hind limbs to Styrofoam working surface
3. ___ Thoroughly spray hindlimbs with IPA
4. ___ Carefully remove the skin of both hindlimbs
 - a. The hindlimb should be exposed from the knee joint to the midpoint of each foot
5. ___ Carefully remove connective tissue around the Tibialis Anterior (TA) Muscle
 - a. Make sure to expose the origin of the EDL at the knee
6. ___ Create a pocket deep to the distal TA tendon below the ankle
7. ___ Cut the distal TA tendon
8. ___ Holding the TA by its distal tendon, pull it towards the knee cutting away connective tissue as needed to separate it from the EDL
9. ___ Cut the TA at its origin and set aside to expose the EDL

10. ___ Create a pocket deep to the distal EDL tendon
11. ___ Cut the Distal EDL tendon
12. ___ Holding the cut tendon, carefully pull EDL towards the knee separating it from the surrounding tissue
 - a. Avoid stretching the muscle as it will damage individual myofibers
13. ___ Cut the proximal EDL tendon
 - a. Tendon to Tendon isolation is critical to maintain fiber integrity
14. ___ Place EDL muscle into 3.0 mL of collagenase solution
15. ___ Repeat process for contralateral hindlimb

Muscle Digestion

1. ___ Place 15 mL conical with EDL muscles and collagenase in 37⁰C water bath
2. ___ Repeatedly invert the 15 mL conical for every 10 min of incubation
3. ___ Incubate for 40 min
 - a. Digestion can take between 50-70 min depending upon collagenase activity
4. ___ Following 50 min, remove 15 mL conical every 5 min and inspect for proper digestion
 - a. Use dissecting microscope light on brightest setting for lighting
 - b. When properly digested individual myofibers should begin to protrude from the surface of the muscle belly
 - c. If the muscle appears fuzzy under a dissecting microscope or individual fibers appear fat and milky in color, the muscle has been overdigested
5. ___ Upon proper digestion level, remove 15 mL conicals with HS and muscles from the water bath
6. ___ Spray conicals down with IPA
7. ___ Remove dish 1 from incubator
8. ___ Coat glass pipettes in HS
9. ___ Use glass pipettes to remove muscles from the 15 mL conical and transfer into the first dish
 - a. Place both muscles into the first dish
 - b. Place collagenase solution back into the incubator (in case further digestion is needed)
10. ___ Use glass pipettes to dissociate muscles
 - a. Progressively use smaller bevel pipettes as the muscle becomes more dissociated
 - b. The muscle should only be washed with media or gently triturated
11. ___ Coat P1000 pipette tips with HS
12. ___ Use a dissecting scope and P1000 pipette to pick out individual live myofibers from the dish
 - a. Live myofibers will appear shiny or clear and be straight or crinkly
 - b. Dead myofibers will appear opaque and bent or short
13. ___ Place all live fibers in the next numbered dish
 - a. Muscles should not remain out of the incubator for more than 15 min
14. ___ Repeat transfer process for the other wash dishes
15. ___ Transfer fibers as described into the collagen and laminin coated dish
16. ___ In the TC hood, add FGF-2 to the dish to a final concentration of 2 ng/mL
17. ___ Place final dish back in the incubator
 - a. Myoblasts should begin to migrate from the myofibers within 3 days
18. ___ Passage cells upon local confluence or average 80% confluence

Notes:
