ELECTRICAL STIMULATION INDUCES AMPK-MEDIATED CELL CYCLE ARREST IN C2C12 MYOBLASTS

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ABSTRACT

Skeletal muscle is a phenotypically dynamic tissue that responds to alterations in activation which can have an affect on muscle recovery from injury Using a cell culture model system, electrical stimulation caused 18 and 12 fold increases in protein levels of p27 and p21, respectively This suggested that electrical stimulation was inducing cell cycle arrest in these cells Thus, we stimulated proliferating myoblasts and examined the effects on p27 protein levels in these cells p27 protein levels increased while cyclin E protein levels decreased with time from 1 to 5 days of electrical stimulation suggesting these cells are undergoing cell cycle arrest Concomitant with these changes was an increase in pAMPK and T198-p27 (a direct AMPK stabilizing phosphorylation site on the p27 protein) Pharmacologic inhibition of AMPK activation using compound C blunted electrical stimulation-induced increases in AMPK activation, T198-p27 and total p27 protein levels These data suggest that electrical stimulation alters the activity of AMPK in C2C12 myoblasts, resulting in cell cycle arrest

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There were many people who played a role in my successful completion of this thesis. Firstly, I would like to thank Dr. Michael Connor for his constant support and help throughout this entire process. I had so much fun in his lab and am so grateful for the skills which I attained in the past 2 years. The past and present Connor lab members also contributed to my project, I would sincerely like to thank each and every one of you for all of your help and advice over the years. Specifically, Jordan Zeppieri and Deep Trivedi helped me a great deal with many facets of my project and made the lab that much more fun to be in! I look forward to our continued friendships. There were many labs which also helped me a great deal; the Ceddia lab, particularly Pinky and Dr. Rob Perry, I have enjoyed the conversations over the years and really do appreciate all the wise advice and sound knowledge you have given to me. The Riddell lab, Hamadeh lab and Hood lab have also helped a great deal whether it be through sharing equipment or ideas. I would also like to thank members of my committee Dr. Ola Adegoke, Dr. Tara Haas, and Dr. John McDermott. Finally I would like to thank my family and close friends who have offered nothing but support throughout the entirety of my academic career, and who continue to support me as I venture off to medical school! Thank you to everyone very much, I really did enjoy myself the past 2 years!

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ABBREVIATIONS

Ab/Am – anti-micotic/anti-biotic

AMP – adenosine monophosphate

AMPK – AMP activated protein kinase

AMPKK – AMPK Kinase

ATP – adenosine triphosphate

bHLH – basic helix loop helix

c-Fos – FBJ osteosarcoma oncogene

CaMKI – calmodulin-dependent protein kinase I

CaMKK – calcium/calmodulin dependent protein kinase kinase

CDC25A – cell division cycle 25 homolog A

CDK – cyclin dependent kinase

CKI – cyclin dependent kinase inhibitor

DM – differentiation media

DMEM – Dulbecco's modified eagle's medium

DNA – deoxyribonucleic acid

EC – excitation coupling

FGF8 – fibroblast growth factor 8

G0 – gap phase 0

G1 – gap phase 1

G2 – gap phase 2

HGF – hepatocyte growth factor

IGF – insulin-like growth factor

IL – interleukin

I.P. – immunoprecipitation

LIF – leukemia inhibitor factor

LKB1 (aka stk11) – serine/threonine kinase 11

M – mitosis

MAPK – mitogen-activated protein kinase

MEF2 – myocyte enhancer factor 2

MHC – myosin heavy chain

MRF – myogenic regulatory factor

Myf5 – myogenic factor 5

MyoD – myoblast determination factor

NO – nitric oxide

P220NPAT – nuclear protein, ataxia-telangiectasia locus

Pax – paired box protein

PGC-1α – perioxisome proliferator-activated receptor gamma coactivator 1

PI3K – phosphotidylinositol 3 kinase

PM – proliferation media

Rb – retinoblastoma

S – synthesis

SAP155 – S-layer protein

SCF-skp – Skp, Cullin, F-box containing complex

SMC – smooth muscle cells

SW1-SNF – Smarca4 Sw1/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4

TGF-β – transforming growth factor beta

1.0 OVERVIEW

Proper differentiation of skeletal muscle is imperative for recovery from muscle injury as well as combating muscle disease Myogenesis is a multistep and complex process involving an array of signalling proteins, expressed at specific points within development Muscle contraction has been shown to be a powerful stimulus for phenotype alterations and a plethora of studies have been completed investigating the effects of muscle contraction on intracellular signalling A common model employed to examine these effects *in vitro* is activation via electrical stimulation of muscle cells in culture This model has been well documented to mimic the effects of muscle contraction in an *in vitro* environment and has been utilized in the past to explore contractile-dependent protein expression alterations responsible for mitochondrial biogenesis [1] Metabolic function [2] as well as cell morphology [3] modifications due to electrical stimulation have also been investigated Although these studies and others similar to them provide proficient insight into the intracellular responses due to electrical stimulation, there is a severe lack of knowledge regarding the effects if any, electrical stimulation has on myoblast proliferation My thesis will systematically evaluate the protein content of multiple myogenic regulatory factors (MRFs) and various cell cycle regulators in response to electrical stimulation *in vitro* to determine the effect, if any, electrical stimulation has on muscle development

2.0 MYOGENESIS

There are two conditions in which myogenesis occurs, the first being embryonically, and the second being in response to muscle injury and disease via satellite cell activation. Embryonic skeletal muscle, in general, is derived from the somite as myogenic progenitor cells. With somite maturity, the myogenic progenitor cells become restricted to the epithelium of the dermomyotome where myogenesis can be induced through the splitting of cells which express myogenic regulatory factors (MRFs) [4]. In the second case, during post-natal muscle growth, after injury or in response to a muscle wasting disease, satellite cells become activated and begin to proliferate. During their proliferation, MRFs as well as members of the Pax (paired box protein) family of regulatory proteins are expressed concurrently. For these cells to then differentiate into fibres the down regulation of the Pax proteins and sequential MRF expression is required [4].

2.1 Embryonic Myogenesis

Understanding the origins of skeletal muscle and how embryonic skeletal muscle differentiation occurs can provide insight into the networks which may promote adult myogenesis via stem cells. The majority of embryonic skeletal myogenic progenitors are formed from somites (transient condensations of the paraxial mesoderm which form around the neural tube) [5]. The somites give rise to the epithelial dermomyotome as well as the mesenchymal sclerotome as

seen in Figure 1. The dermomyotome gives rise to the dermis as well as skeletal muscle of the trunk and limbs where the mesenchymal sclerotome ultimately develops into cartilage and bone of the vertebrae and ribs [5]. Myogenic precursors in the dermomyotome express Pax3, Pax7 and low levels of Myf5 (myogenic factor 5) (all to be described later). Furthermore, deep back muscles are formed when progenitor cells of the dorsal medial lip give rise to the epaxial myotome, again shown in Figure 1. Similarly, the ventral lateral lip generates the ventral non-migratory hypaxial myotome which is responsible for lateral trunk muscle development. Ventral lateral lip cells also separate form the dermomyotome and develop to form muscles of the limbs, ventral body wall, diaphragm and tongue [5].

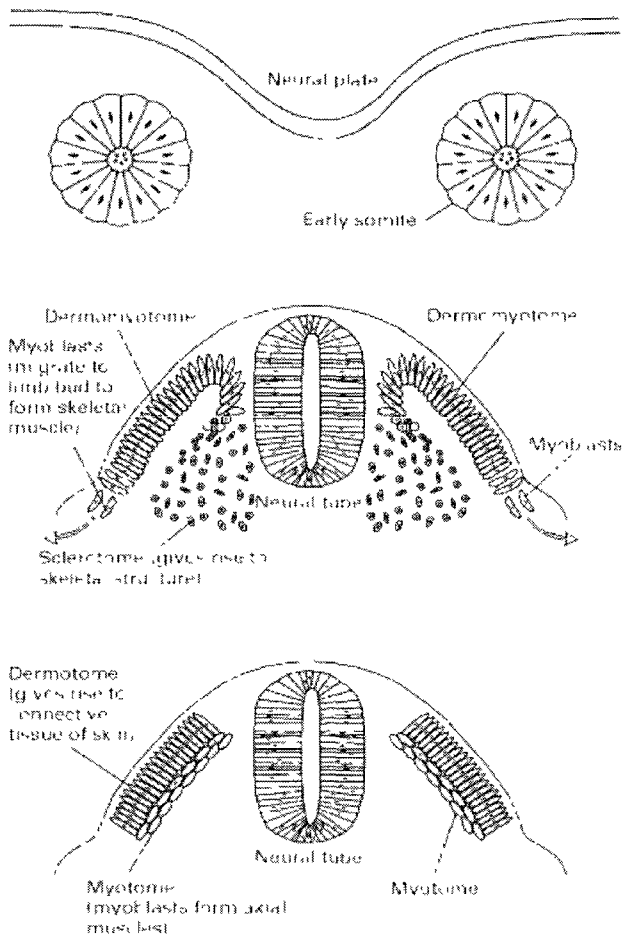

Figure 1. Stages of embryonic myogenesis. A. Somites are formed after neural plate formation. B. Each somite gives rise to a dermomyotome which gives rise to skin and muscle as well as a sclerotome. The sclerotome is responsible for forming skeletal structures. C. Finally the dermotome gives rise to connective tissue and the myotome gives rise to axial muscles. Adapted from M. Buckingham, 1992, *Trends Genet.* 8:144.

Embryonic myogenesis relies primarily on the expression of two gene families, namely the MRF and myocyte enhancer factor 2 (MEF2) families MRFs describe proteins belonging to the super family of basic helix-loop-helix (bHLH) transcription factors [6-9] The MRF family consists of MyoD (myoblast determination protein), myogenin, Myf5, and MRF4 (myogenic regulatory factor 4) all of which share approximately 80% of their amino acid identity and a bHLH motif responsible for dimerization and DNA binding [8] MRFs can form heterodimers with E proteins (ubiquitous bHLH proteins) by binding to the E-box consensus sequence [CANNTG] in muscle gene promoters and enhancers [7 8] Although all of the MRFs bind to the same DNA sequence with similar affinity, the expression pattern of each MRF is distinct from one another In general, it is understood that MyoD and Myf5 are expressed in proliferating myoblasts, while myogenin and MRF4 are expressed in cells which have exited the cell cycle typically due to mitogen depletion [8] Figure 2 shows the myogenic process with expression of MRFs at specific differentiation points Embryonically, Myf5 activation occurs first around 8 days postcoitum (dpc) and begins to be down regulated after 14 dpc [10] Myogenin activation is next occurring at 8 5 dpc followed by MyoD at 10 5 dpm concurrent with other markers of terminal differentiation MRF4 finally is momentarily expressed between days 9 and 12, then repressed until birth [11] Not only do MRFs regulate the transcription of muscle specific genes, they also have the ability to auto regulate each other's

transcription. MyoD is capable of positive auto regulation as well as inducing myogenin expression, which in turn induces MyoD expression [12]. This implicates MyoD and myogenin to be constituents of an auto regulatory loop for the purpose of amplifying expression levels of these two proteins to threshold levels required to induce the myogenic process. Additionally, MyoD is capable of inhibiting Myf5 expression [13], while MRF4 is required for down-regulation of myogenin [14], as seen in Figure 2.

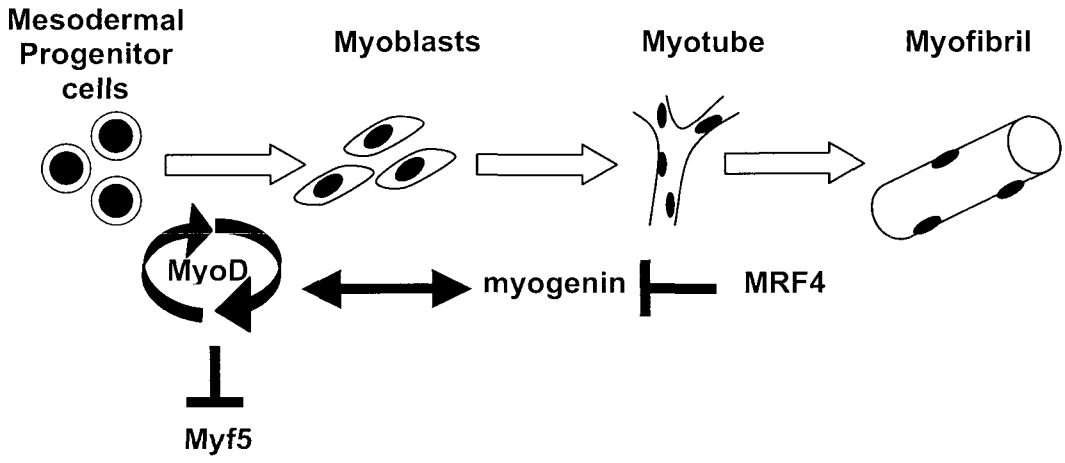

Figure 2 The myogenic process beginning with mesodermal progenitor cells, which ultimately differentiate into fully functioning and multinucleated myotubes and finally structurally sound myofibrils When mesodermal progenitor cells are determined to become myoblasts through means of growth environment or otherwise, the expression of the primary MRFs, Myf5 and MyoD allow differentiation into myoblast cells MyoD has autoregulatory capabilities and can also inhibit Myf5 Myoblasts are capable of differentiating into myotubes with the expression of the secondary MRFs myogenin and MRF4 MyoD and myogenin cause each others expression to increase, while MRF4, which is required for fusion and formation of myofibrils, down-regulates myogenin expression

Forced expression of any one MRF is adequate to induce myogenesis even in a variety of non muscle cells in culture [15 16] MRFs have also been shown to cause direct activation of the MEF2 family of MADS box factors, which is required to convert cells to skeletal muscle [15 17] In humans, four versions of the MEF2 gene exist denoted as MEF2A, MEF2B, MEF2C, and MEF2D, all of which are distinctly expressed with overlapping arrangements during embryogenesis through adulthood [17] MEF2 genes however, are unable to sufficiently induce myogenesis unlike members of the MRF family The activation of transcription of muscle specific proteins is most efficient when MRFs and MEF2 family members act in a synergistic manner, as the binding of one factor to DNA helps to recruit and stabilize the binding of the other factor [17]

Investigation into the necessity of each MRF in embryogenesis has been completed mainly through null or mutated mice models Early research has shown that MyoD is not essential for myogenesis in the mouse embryo MyoD null mice have been found to be fully viable lacking any obvious muscle abnormalities Molecularly, mice lacking MyoD had an increased expression of Myf5 mRNA levels, which provided evidence for a reciprocal type of regulation between these genes [18] Additionally, this study found that both myogenin and MRF4 mRNA levels were also normal compared to MyoD positive mice A later study completed in the C2C12 myoblast cell line however, determined that MyoD was in fact necessary for myoblast fusion, even when endogenous levels of Myf5 and myogenin are high [16] MyoD also has cell cycle inhibition qualities

through cross talk with cell cycle regulators that contain an E box (i e $p21^{Cip1}$) [19] Initial insight into the functional relevance of Myf5 revealed that mice homozygous for a Myf5 mutation had visibly normal skeletal muscle development, however died due to the lack of the distal parts of the ribs, creating an inability to respire [20] These findings are further evidence for the compensatory role which exists between MyoD and Myf5 in regulating skeletal muscle development Later investigation into Myf5 and MRF4 absence revealed that in mice lacking these proteins, the early myotome does not form resulting in the misallocation of cells [21 22]

Contrary to findings from MyoD and Myf5 single knockout models, animals lacking myogenin exhibit severe skeletal muscle inadequacies Previous studies employing myogenin mutant animals found that although the animals survived fetal development, they died perinatally containing far fewer muscle fibres than wild type animals The fibres however did appear to be normal on a gross level, suggesting myogenin is not required for positional development [8 23] Further research into the myofibers present in these animals however, found that on a molecular level, the fibres were extremely atypical Muscle areas were occupied only by sparse myofibers as well as mononucleated cells, negative for myosin heavy chain, indicating the cells are undifferentiated and have an inability to fuse The mutants also expressed decreased levels of MRF4, with normal MyoD expression [8 23] Much investigation into the embryogenic development of muscle cells have facilitated knowledge growth in this area Research observing the

second method of myogenesis, namely via satellite cell activation, has also been very successful in efforts to expand knowledge

Additionally, there is a very prominent negative regulator of skeletal muscle growth, namely myostatin Myostatin is a member of the transforming growth factor- β (TGF-β) family of growth factors Myostatin is initially expressed in the myotome and remains to be expressed at varying levels within skeletal muscle through adulthood. however is undetectable in most other tissues Animals lacking the myostatin gene, or animals with even a 25% disruption in the gene experience larger weight and muscle mass that control animals[24] Where the MRF and MEF2 family promote muscle growth and differentiation, myostatin is a prominent inhibitor of muscle growth [24]

2 2 Satellite Cell Activation-Dependent Myogenesis

Satellite cells were initially seen in frog skeletal muscle described only by their morphology in mature muscle fibres [25] Satellite cells lie on the periphery of muscle fibres between the sarcolemma and the basal lamina (hence the name "satellite" cells) and form a stable and self-renewing population of stem cells within the adult muscle [26] [27] [28] In the adult, satellite cells mediate the growth of muscle tissue as well as recovery of the cells post-trauma Initially, satellite cells were thought to be predisposed to become myogenic precursor cells, however studies have shown that myoblasts (C2C12 cells) have the ability to become osteogenic and even adipogenic in the appropriate growth conditions [29] [30]

These data lead to the view that satellite cells are in fact multipotent

There are multiple criteria which allow proper identification of satellite cells Cell morphology is obviously important, that is their localization between the basal lamina and the sarcolemma of myofibrils Additionally satellite cells contain a high nuclear·cytoplasmic ratio, high heterochromatin content and low cytosolic organelle content [26-28] A common protein marker of satellite cells, Pax7 transcription factor, is expressed in the nuclei of quiescent as well as activated satellite cells [26] When Pax7 null mice were investigated, the only difference found between the null mice versus wild type mice was the lack of satellite cells [26 28] In embryonic muscle development, members of the bHLH family of proteins (MRFs) regulate the transition from one stage of development to the next, quiescent satellite cells of adult muscle do not express these proteins until after satellite cell activation There is an abundance of data which focuses on satellite cell origin as well as embryonic satellite cell development, however this is out of the scope of my thesis What is relevant however, is the activation process of the satellite cells as well as the mechanisms which allow these cells to repair muscle damage

There are a variety of stimuli which have been shown to cause activation of quiescent satellite cells including physical load, stretching, damage, electrical stimulation, and degenerative diseases such as muscle dystrophies [26 28] Satellite cell activation is also not as simple as site dependent activation In fact, damage to one end of a single myofibril activates satellite cells throughout its

entirety These cells then enter the cell cycle and eventually give rise to myogenic precursor cells These precursor cells are now determined to become muscle cells, albeit after key stages of muscle differentiation The number of quiescent satellite cells in adult muscle is virtually constant through multiple stages of regeneration, implying these cells contain an inherent self-renewal quality [28] There exists much data revealing the satellite cells' ability to self-renew, however, relevant to my thesis is the differentiation of these satellite cells into muscle tissue

As mentioned previously, what commits satellite cells for myogenic differentiation is their growth environment In particular, hepatocyte growth factor (HGF) [31] as well as other growth factors such as fibroblast growth factor (FGF), transforming growth factor beta (TGF-β), insulin-like growth factor (IGF-1), interleukin-6 (IL-6), leukemia inhibitor factor (LIF) and nitric oxide (NO) [28] are all factors that regulate the proliferation as well as differentiation of satellite cells HGF is a major regulator of satellite cell response during muscle regeneration HGF has been shown to independently induce satellite cell activation *in vitro*, and has additionally been shown to aid in the migration of satellite cells to injured areas of the myofibril [31] [32] There appears to also be a cause and effect relationship between HGF and NO NO is released from beneath the basal lamina after damage has occurred The NO released then causes HGF release from the extracellular matrix [33] The FGF family members have been implicated in controlling many molecular events related to myogenesis as well In

particular, FGF-6 is involved in the mediation of early muscle growth via satellite cell activation [34] FGF-2 appears to also have importance in this story as this growth factor has been previously detected in the basal lamina which envelopes developing and mature myotubes [35] IGF-I and IGF-II are well known to be involved in the regulation of muscle growth and repair as well as in satellite cell proliferation High IGF-I levels have been repeatedly implicated in muscle hypertrophy; this hypertrophy is attributed in part, to satellite cell proliferation and differentiation [36] The TGF-β family has regulatory components involved with satellite cell myogenesis, as previously described myostatin is a member of the TGF-β family, which has inhibitory effects on muscle growth [26] Once satellite cell proliferation occurs, the appropriate growth conditions, that is presence of particular growth factors, will allow determination of the cells to the myogenic form Post activation, the cells express members of the bHLH family and proceed with differentiation until viable myofibrils are formed, or until muscle repair has occurred

Embryonic myogenesis is similar to satellite cell mediated myogenesis in many ways The initial stages of regulation of muscle development regulated by bHLH family members as well as later muscle formation are consistent between myogenic methods There are however key differences between the two methods, specifically concerning the role of the MRFs Quiescent satellite cells have low Myf5 expression, with no other MRF expression Additionally, the expression order of the MRFs following satellite cell activation differs from

embryonic MRF expression. MyoD is the first to be expressed, followed by Myf5 and MRF4, however myogenin does not become synthesized until myosin heavy chain (MHC) begins to be expressed [26]. Although there are differences between embryonic and satellite cell dependent forms of myogenesis, the result of both is fully functioning differentiated muscle tissue.

2.3 Mature Muscle Fibres

Whether it be embryonically or through means of satellite cell activation, the result of these processes is the formation of mature muscle tissue after fusion of myotubes. There are three types of muscle classified as smooth, cardiac, and skeletal. The relevance of my thesis lies within skeletal muscle physiology, however it is important to also have an understanding of how the other types of muscle work as well as the differences and similarities between all types of muscle.

Skeletal muscle is a voluntary and striated form of muscle, which occupies 40% of human body weight[25]. The main function of skeletal muscles is to stabilize the skeleton as well as provide a method for mobility. The structural components of the sarcomere (functional component of muscle fibre) can be seen in Figure 3. There has been much investigation into the mechanisms of muscle contraction over the past century, however the basic understanding of how muscle contracts is now well established. At the beginning of the 20th century the accepted theory of muscle contraction was the "lactic acid

theory", which held that lactic acid-induced folding of long protein chains along muscle fibres caused muscle contraction. This was later disproved by the completion of an experiment which saw muscle contraction even in the absence of lactic acid [37]. Although disproved, the lactic acid theory was the primary theory of muscle contraction until the early 1950s. At this point Huxley suggested that contraction occurred through the relative sliding of two sets of filaments [38] and he later confirmed his theory [39]. Once the sliding filament theory was confirmed, the theory was furthered by the development of the cross-bridge theory for the mechanism which causes the sliding of the filaments[40]. Since the postulation of the cross-bridge theory, there has been much investigation into the exact mechanisms of this process, and many alterations to the original theory have formed the current model for cross-bridge formation and the sliding filament theory.

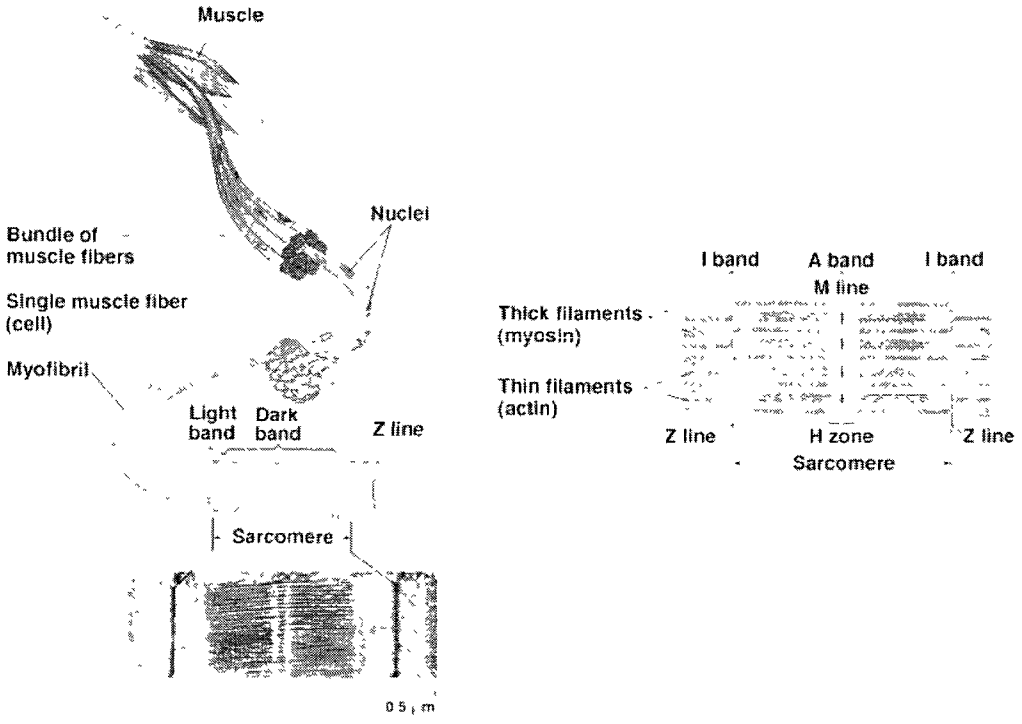

Figure 3. The Sarcomere – the functional component of a muscle fiber. Sarcomere is defined as Z line to Z line. The thick and thin filaments work together cause a shortening of the muscle (contraction). Adapted from Addison Wesley Longman, Inc, 1999.

The sliding filament theory based cross-bridge theory is the paradigm used currently to describe current mechanisms of muscle contraction. The modern theory shows myosin-based cross bridges that attach to actin and pull the actin filament towards the centre of the sarcomere, causing a shortening of the muscle tissue (contraction) and thus force [41]. However, what signalling mechanisms cause this cross-bridge formation and subsequent muscle contraction?

Initially, an α-motor neuron provides an action potential to a motor unit (α-motor neuron and the muscle fibres it innervates). If the action potential is of sufficient magnitude (reaches threshold), depolarization of the fibre membrane occurs. This depolarization leads to muscle contraction; the idea which links membrane depolarization and muscle contraction is excitation-contraction (EC) coupling [42]. The action potential travels the length of the fibres as well as down the fibre T-tubules. The T-tubules in skeletal muscle lead to the sarcoplasmic reticulum which releases calcium in response to an electrical gradient. The released calcium binds to a protein troponin, which at rest covers the active site on the actin molecule. The binding of calcium to troponin reveals the active site of actin to allow for myosin to bind to it, thus forming a cross-bridge and subsequent muscle contraction.

Cardiac muscle is also a striated muscle type, however it is involuntary. Unique to cardiac muscle is its own regulatory component for frequency of muscle contractions. Although interesting, I will be focusing on the

contraction mechanisms alone for cardiac muscle contraction. Mechanisms of contraction within cardiac muscle are similar to skeletal muscle mechanisms; that is the model for contraction is also EC coupling. As within skeletal muscle, the basic contractile unit within a cardiomyocyte is the sarcomere [43].

Smooth muscle is unique from both skeletal and cardiac muscle as it is not striated, its appearance is "smooth". This type of muscle is also involuntary, that is, smooth muscle receives neural innervation from the autonomic nervous system. Smooth muscle is found in many areas of the body, in particular research has focused on the muscle lining the airway, blood vessels and the intestine. Although muscle plasticity as well as function varies greatly in smooth, cardiac and skeletal muscle, they all still utilize actin-myosin interactions to contract. [44, 45]. What differs with smooth muscle is that the muscle contractile state is regulated by various hormones as well as autocrine and paracrine actions [45]. Although the specific mechanisms involved with smooth muscle contraction aren't specifically apparent to my thesis, it is helpful to outline that there are large differences between all types of muscle, thus molecular signalling mechanisms which may be occurring one muscle type may not be occurring in the others.

3.0 THE CELL CYCLE

Cell division is a major process in muscle differentiation and satellite cell activation In order for proper division to proceed two successive processes must occur DNA must be doubled and then subsequently split into 2 equal and identical fractions In order for these processes to occur, the cell is guided through the cell cycle There are four phases within the mammalian cell cycle, namely. G1 (gap phase 1). S (synthesis phase). G2 (gap phase 2), and M (mitosis phase) [46] [47] During G1 cells are generally increasing in size and preparing for DNA synthesis Replication of the DNA and thus doubling of the genome occurs in the synthesis phase G2 is a phase with the purpose of checking DNA integrity and preparing the cell for division (M phase) There is another stage known as G0, which is a quiescent stage of non-growth Cells can enter G1 from G0 before they are committed to replication [46] [47] [48] There are however, external regulators (i e growth factors) which influence a cell's progression through this cycle In addition, checkpoints exist to ensure the integrity and purity of the copied DNA material and the production of identical cells within that population Additionally, there are also regulators which act on the cell cycle by slowing or speeding its rate of progression Accelerating the progression through the cell cycle are active cyclin/cyclin dependent kinase (CDK) complexes and their associated inhibitor families (members of the INK4 and Kip families) [46] That is, when the INK4 or Kip family members bind to the cyclin-CDK complex, cell cycle inhibition occurs Figure 4 shows a summary of

these cyclin/CDK complexes as well as where they affect the cell cycle.

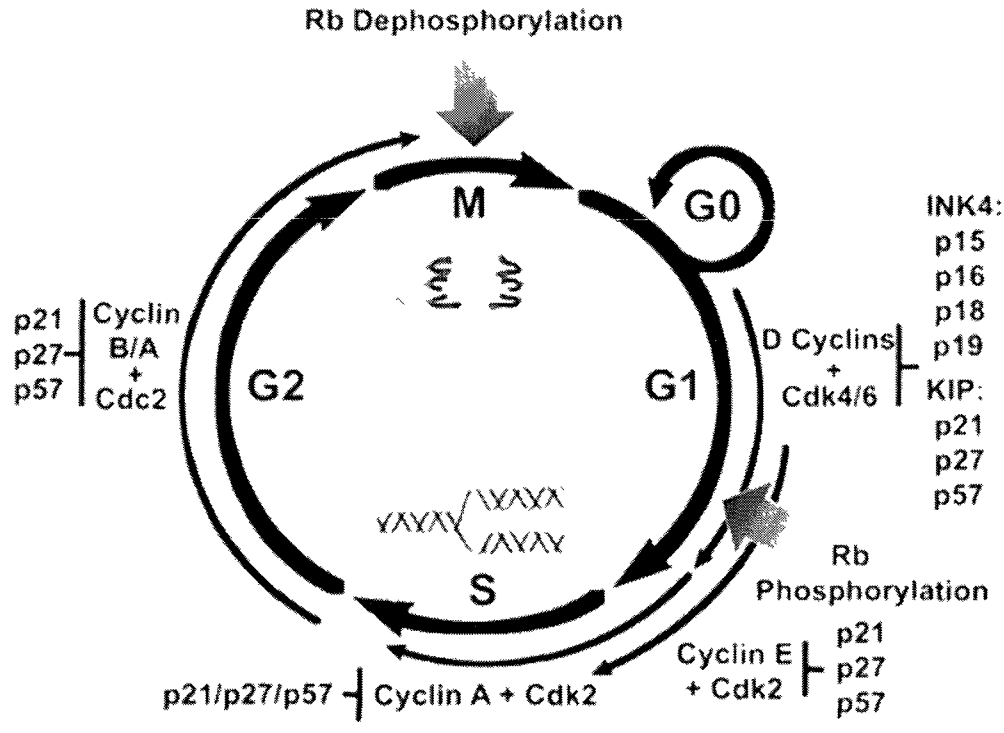

Figure 4. Stages of the cell cycle. At the G1/S phase transition, the cyclin E/CDK2 complex is responsible for pushing the cell into S phase past this stage's checkpoint. Members of the KIP family are CKI's for the cyclin E/CDK2 complex and inhibit the cell's progression through the cell cycle. Adapted from Donovan and Slingerland *Breast*

3 1 Cell Cycle Progression

Cyclin proteins are expressed in a cyclical manner, whereas CDK protein expression stays relatively constant throughout the cell cycle CDKs are a family of serine/threonine protein kinsases that become activated at different parts within the cell cycle depending on the cyclin being expressed at that point [47] There have been nine CDKs identified, however only five of these have influence on the cell cycle, specifically, during G1-(CDK4, CDK6 and CDK2), S-(CDK2), G2- and M-(CDK1) phases In order for the cell cycle to progress, cyclins must form active complexes with their associated CDKs and expression levels of their respective inhibitors must be at a low enough level not to impede cell cycle progress Relevant to my thesis is specifically the G1 to S phase transition At this point a major checkpoint referred to as the restriction point occurs and the cell is preparing for DNA replication However, in order to understand the complexities which occur in the G1 to S transition, the mechanisms involved with cell cycle entrance (G0 to G1 transition) must be elucidated

3 1 1 G0/G1 Transition

For entrance into G1, the cyclin D isoforms (Cyclin D1, D2, and D3) must be expressed and bind to CDK4 and to CDK6 [46 48] Cyclin D is quite unique as it is the only cyclin which is not expressed periodically but is synthesized for as long as mitogen stimulation persists There are many factors which stimulate production of cyclin D, including mitogen-activated protein kinase

(MAPK) [49], phosphotidylinositol 3 kinase (PI3K) [50], fibroblast growth factor 8 (FGF8) [51] and FBJ osteosarcoma oncogene (c-Fos) [52] to name a few

There has been investigation into the importance and function of cyclin D, CDK4 and CDK6 Knockout models have shown that the requirement for CDK4/6 is tissue specific, however it appears as though the absolute requirement for CDK4/6 is questionable [47] CDK4 null mice are viable, however are under sized and there are tissue specific defects that ultimately lead to diabetes and infertility The cell cycle specific defects were investigated in CDK4 deficient mouse embryonic fibroblasts and it was found that the cells did grow, however entrance into the cell cycle from quiescence was delayed [53] CDK6 null mice are similarly viable, however these mice are of normal size Tissue specific defects from CDK6 gene deletion include splenic and thymic hypoplasia [54] There is compensation between these two CDKs as deletion of either gene still generates a viable animal Cyclin D null mice are also viable but, similar to CDK4 mice, are undersized [55] It appears as though there may be some degree of a compensatory role between the cyclin D isoforms as well [56] Cyclin D1 null mouse cells are still able to progress through the cell cycle, however some in vivo developmental irregularities have been shown to occur neurologically [57] Mice lacking the cyclin D2 or cyclin D3 gene also appear to have developmental issues specific to tissue type [58] [59]

Cyclin D has two main purposes within the cell cycle to phosphorylate retinoblastoma (Rb) and to sequester Kip proteins Rb is a tumour

suppressor that was first discovered in a retinoblastoma cancer The Rb protein is responsible for a pivotal G1 checkpoint (restriction point) blocking S phase entrance [60] Rb is either in a hypo- or hyper-phosphorylated state, when hyperphosphorylated Rb dissociates from E2F transcription factors (imperative for DNA synthesis) and is targeted for degradation [61] When hypophosphorylated, Rb inhibits cell cycle progression After Rb phosphorylation, Cyclin D then can be bound by members of the Kip family which causes cytosolic translocation and sequestering of Kip protein members to allow for Cyclin E expression, and thus Cyclin E related events Cyclin D then remains in the cytosol for the remainder of the cell cycle, until division and re-entrance into G1 is required [62]

Specifically inhibiting the effects of the active cyclin D CDK4/6 complex are members of the INK4 family, particularly $p16^{INK4A}$, $p15^{INK4B}$, $p18^{INK4C}$, and $p19^{INK4D}$ Each member of the INK4 family are capable of binding CDK4 and CDK6 which prevents cyclin D from binding to the CDK, therefore inhibiting activation of a cyclin-CDK complex [46] Biochemically, members of the INK4 family behave identically with respect to the cell cycle, although differences with respect to their effects on cancers have been identified [46]

3 1 2 G1/S Transition

Cyclin D does play a role in G1/S phase transition, however cyclin E and its specific CDK (CDK2) are responsible for the majority of the kinase activity For many years it was believed that CDK2 was critical for progression and exit of

S phase It was found however, in some human colon cancer cell lines, that CDK2 was not necessary for survival [63] This lead to in vivo investigation of CDK2 null mice viability Two groups found that these CDK null mice were viable yet experience both male and female sterility Compared to other cyclin or CDK single knockout models, the CDK2 knockouts were actually the least compromised, having survived up to 2 years free from any pathology (other than sterility) [64] It has been recently found that although CDK2 plays a pivotal role in S phase transition, CDK1 plays a compensatory role when CDK2 is knocked out [65] Cyclin E exists in two isoforms cyclin E1 and cyclin E2 Deletion of cyclin E1 in vivo resulted in normal animal phenotype, whereas deletion of cyclin E2 resulted only in a reduction of male fertility However in a double knockout model of cyclin E1 and cycin E2, embryonic death occurred [66] It is interesting that the CDK2 null mice models were relatively viable due to CDK1 compensation, whereas when both cyclin E1 and E2 are knocked out the animal is not viable, inferring no compensatory role from other cyclins This leads to the suggestion that the necessary functions of cyclin E are not dependent solely on CDK2

Unlike cyclin D, cyclin E expression is cyclical Typically, cyclin E becomes expressed during G1 in response to cyclin D expression Cyclin E levels then begin to drop in S phase as cyclin A levels rise and finally cyclin E proteins are essentially undetectable in G2 [66] Due to the cyclical nature of cyclin E, the kinase activity of the cyclin E/CDK2 complex is periodic and reaches a maximum at the G1/S phase transition [67] There are similarities in the expression and

targets of cyclin E compared to cyclin D proteins Specifically, cyclin E expression and activity is in part sensitive to mitogen presence and cyclin E has downstream targets which are shared by cyclin D (Rb and p27); however, the mechanisms and result of phosphorylation on these targets is different from cyclin D

The cyclin E/CDK2 complex does not sequester p27, rather phosphorylates the p27 not bound by cyclin D, on the threonine 187 residue [66 68] This phosphorylation provides for a recognition motif for Skp, Cullin, F-box containing complex (SCF^{Skp2} ,an E3 ligase), which targets the p27 protein for ubiquitin-dependent degradation [66 69] In this sense, cyclin E promotes its own expression through decreasing the protein levels of its inhibitor As mentioned previously, cyclin E/CDK2 phosphorylates Rb but on different sites than cyclin D/CDK4/6 The phosphorylation of Rb leads to dissociation from E2F (as described earlier) which results in cyclin E transcription Therefore, cyclin E/CDK2 activation not only inhibits its inhibitor, but also promotes its own transcription The phosphorylation effects of cyclin E/CDK2 described thus far are primarily G1 phase cell cycle effects Cyclin E/CDK2 further phosphorylates a second group of substrates which are involved more directly with cell duplication [66] Specifically, cyclin E – associated kinases have been implicated in the phosphorylation of nucleophosmin, CP110, p220NPAT (nuclear protein, ataxia-telangiectasia locus), E2F-5, p330/CBP, SWI-SNF complex (SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a,

member 4), SAP 155 (S-layer protein) and CDC25A (cell division cycle 25 homolog A). Phosphorylation of these targets contributes to varying results including centrosome duplication, histone biosynthesis, gene expression control, pre-mRNA splicing and cell cycle progression [70]. Although the precise mechanisms of phosphorylation as well as the specific results of those phosphorylations are important, they may be beyond the scope of my thesis.

As seen in Figure 4, inhibitors of the cyclin E/CDK2 complex are members of the Kip family, specifically p21, p27 and p57. Members of the Kip family have the ability to inhibit all cyclin/CDK complexes within the cell cycle. In a quiescent cell p27 levels are relatively high, whereas in cycling cells essentially all of the p27 molecules are associated with cyclin D/CDK4/6 [71]. p27 is widely considered to be a key regulator of the G1/S phase transition where p21 plays a role in p53-mediated cell cycle arrest [46]. It is common to use p27 as a prognostic tool in cancer physiology, that is, when a tumour is p27 negative the prognosis is poor and when a tumour is p27 positive the prognosis is good. p27 is also the only member of the Kip family which has direct and mutual phosphorylation actions with the cyclin E/CDK2 complex. As previously mentioned cyclin E/CDK2 can phosphorylate p27, promoting it for degradation; on the other hand p27 can directly bind to the cyclin E/CDK2 complex inhibiting its cell cycle progression activities. For this reason, p27 and cyclin E levels prove to be a beneficial indicator of the cycling status of the cell.

4.0 MYOGENIC REGULATION VIA THE CELL CYCLE

As previously mentioned, a prerequisite for myogenesis is cell cycle cessation. Myoblasts will continue to proliferate until eventual molecular signalling leads to cell cycle arrest. Once quiescent, differentiation to myotubes can occur. In this sense, alterations affecting the cell cycle can lead to a premature or delayed differentiation of myoblasts which ultimately leads to abnormal muscle development. In the cancer field much research has been completed investigating possible master regulators of cell cycle arrest. Implicated as having a role in cell cycle cessation is AMP activated protein kinase or AMPK. Although most examination of AMPK has occurred in the metabolism field, this versatile protein has cell cycle regulatory effects. As muscle is a very metabolically active tissue which contains high amounts of AMPK, it is logical that these AMPK mediated cell cycle effects would be taking place in myoblasts.

4.1 AMPK signalling

AMPK is a serine-threonine kinase which is known for being involved in the regulation of cell metabolism [72]. Structurally, AMPK is a heterotrimeric protein complex: the α-subunit is catalytic, whereas both the β and γ subunits are regulatory. AMPK phosphorylation on Thr172 and thus activation due to ATP depletion, signals the cell for conservation and generation of ATP. AMPK appears to have multiple methods of activation, however is consistently activated by AMPK kinases (AMPKK). Initially it was postulated that AMPKKs could

activate calmodulin-dependent protein kinase I (CaMKI), which in turn can activate AMPK [73]. At the time, it was thought that CaMKI kinase was very unlikely to be an important AMPKK due to low enzyme activity, however this assumption was later shown to be inaccurate.

The tumour suppressor LKB1 was established as an AMPKK through co-purification, immunoprecipitation, and embryonic fibroblast studies [74]. However, LKB1 is not the only AMPKK which is responsible for AMPK activation as studies in HeLa cells (which lack LKB1) still experience AMPK phosphorylation on Thr 172 [75]. With this discovery, focus shifted to Ca^{2+} / calmodulin dependent protein kinase kinase (CaMKK). CaMKK emerged as an important AMPKK through studies on HeLa cells. When CaMKK is inhibited, AMPK activation is severely inhibited, if not completely abolished in the HeLa cells [76]. Based on these data, there are two clear and congregating mechanisms of AMPK activation. The first is LKB1 dependent, which relies on an increase in the AMP:ATP ratio. The second is CaMKK dependent, which is utilized when an increase in intracellular Ca^{2+} occurs. The two mechanisms for AMPK activation can be seen in Figure 5.

Once activated, AMPK provides for a plethora of effects including glycogen synthesis, fatty acid oxidation and protein synthesis. In addition to these metabolic effects AMPK also has cell cycle effects. AMPK has been found to cause cell cycle arrest in hepatoma HepG2 cells [77], mouse embryonic fibroblasts [78], human aortic smooth muscle cells (SMCs) and rabbit aortic strips [79]. A model developed by Motoshima et al in 2006 attributes the cessation of

cell proliferation by AMPK to p53 and subsequent p21 activation, both of which are cell cycle inhibitors. It has been found that the LKB1-AMPK pathway plays another key role in cell cycle regulation. Liang et al in 2007 showed that AMPK directly phosphorylates p27 on residue T198, which allows a direct link to be made between a energy sensing pathway and cell cycle regulators. Although the AMPK pathway is a popular focus for metabolism and cancer research, its contribution to muscle development progression is lacking.

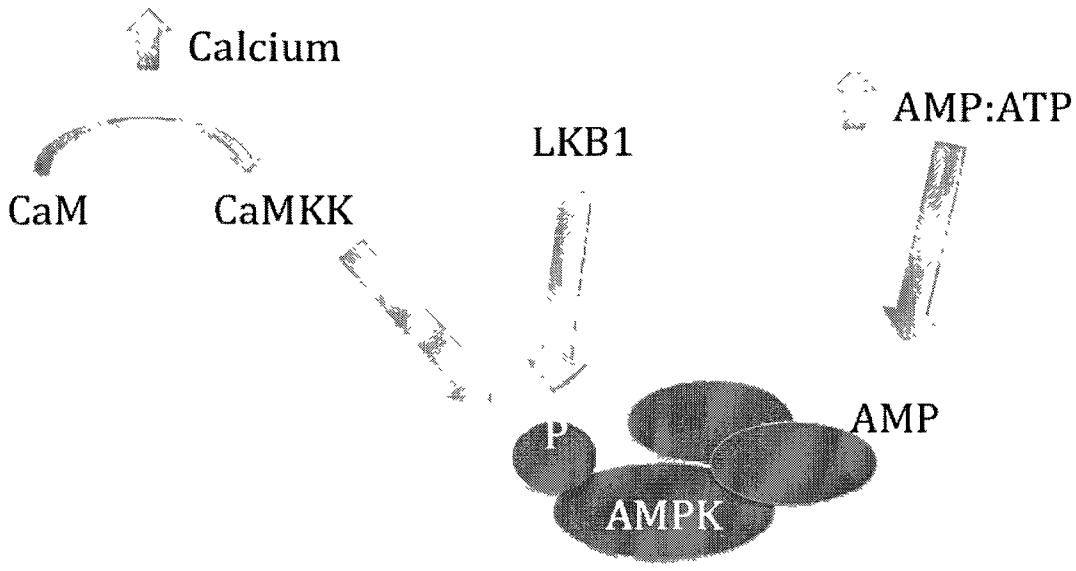

Figure 5. Two methods by which AMPK can become activated. The first method is through an increase in the AMP:ATP ratio. AMP binds to AMPK which allows the AMPK molecule to increase its affinity for LKB1 to phosphorylate the AMPK molecule. The second method is through an increase in intracellular calcium, which activates CAMKK, which subsequently phosphorylates AMPK.

What is known about AMPK and its role in skeletal muscle is primarily metabolic, however there have been some investigation into a possible role within myogenesis. AMPK has been shown in the past to regulate myoblast differentiation caused by glucose restriction through regulation of Nampt [80], a cytokine. More recently, AMPK has also been shown to inhibit myoblast differentiation, however through a PGC-1α mechanism [81]. This same paper looked at p21 as a possible cell cycle target for AMPK via p53, however p53 as well as p21 levels stayed relatively constant during nutrient withdrawal treatment, indicating this is not the cell cycle affecter causing an arresting of the cells. Although AMPK may act to alter PGC-1α expression thus transcription of differentiation necessary mitochondrial related proteins, the cell cycle must still be arrested before differentiation can occur. p21 is often a target protein to investigate within myogenesis as it contains an E-box in its promoter region (recall: MyoD binds E-box proteins). This piece of literature shows however, that possibly p21 is not the CKI responsible for cell cycle arrest due to AMPK regulation. It could be that p27 is responsible for the AMPK mediated cell cycle arrest through T198 phosphorylation.

5.0 ELECTRICAL STIMULATION

Many methods have been developed to analyze the effects of exercise on muscle cells A commonly utilized *in vitro* method is electrical stimulation This treatment is preformed by using 6-well cell culture plate lids Each well contains 2 electrodes which penetrate into the media of the well These plates can then be connected to a power source, where the intensity, frequency and duration of electrical stimulation can be set This method has been used very successfully in culture to analyze effects of electrical stimulation on mainly metabolic processes Electrical stimulation has successfully been implicated in increasing cytochrome c and ATP synthesis [1] Additionally, the differentiation status of myoblast cells has been researched in response to electrical stimulation There is definite evidence supporting electrical stimulation as having an affect on the differentiation status of myoblast cells Previous work has focused on cell morphology [3], scaffolding [82], elongation and alignment [83], as well as myogenic factors such as MRFs and myosin heavy chain [84] Park et al investigated extracellular matrices as well as sarcomere differentiation in myofibrils They showed that despite no change in apoptotic activity, collagen deposition was reduced, while excitability was increased Despite these results the authors do state it is desired for the muscle to have increased cellularity rather than increased extracellular matrix proteins, however this study did not investigate these results further [3] They did find however, that myofibrils subjected to electrical stimulation contained more well developed sarcomeres than

control cells. Jun et al further investigated the potential for electrically conductive composite fibres as a tool in muscle tissue engineering and found that the electrical stimulation fibres increased markers of muscle development, specifically myogenin, troponin T and myosin heavy chain [84]. They further suggested that these substrates could modulate the myoblast to myotube transition.

Although these studies provide for some insight into electrical stimulation effects on muscle development, focus has been directed mainly at rates of transcription for tissue regeneration. There has been no insight into the molecular signalling responsible for the electrical stimulation effects seen previously. Additionally, a complete story outlining the effects of electrical stimulation on the myoblast to myotube differentiation process has yet to be investigated. Electrical stimulation has been investigated as a possible method for muscle tissue regeneration [85], however the possible effects electrical stimulation has on cell cycle progression remain to be outlined.

6.0 STUDY RATIONALE

As previously discussed electrical stimulation has been used as a successful tool for investigation of muscle metabolism as well tissue engineering possibilities. There is currently no data suggesting the role if any electrical stimulation has on the myogenic process as well as the cycling status of these cells. The aim of this study is to examine the various effects of electrical stimulation on proliferating and arrested C2C12 myoblasts from a cell cycle perspective. Various molecular signalling pathways will be examined in an attempt to more fully understand the gambit of molecular influences affecting muscle development. These data are relevant in discovering mechanisms which help or hinder myogenesis. In particular these data will lead to the knowledge of how varying days of exercise may effect satellite cell dependent myogenesis post trauma.

HYPOTHESES

After subjecting C2C12 myoblasts to electrical stimulation, I hypothesize that two separate, yet related results will occur:

1. Electrical stimulation of myoblasts will modulate the abundance and phosphorylation of critical cell cycle regulators leading to cell cycle arrest and;
2. This cell cycle exit will increase the rate of myoblast differentiation via an increase in myogenic related proteins.

METHODS

All experiments were completed under both proliferating and differentiating conditions using the C2C12 myoblast cell line.

Cell Culture

For proliferation experiments, cells were maintained in Dulbecco's Modified Eagle Medium (DMEM), 10% Fetal Bovine Serum (FBS), and 1% Anti-micotic/Anti-biotic (Am/Ab) (to be referred to as PM) at 37°C and 5% CO_2. Prior to plating, the wells of a six well plate were coated with 0.1% gelatine and exposed to UV light for 20 minutes. The plates were coated with gelatine in order to ensure the myoblasts would remain adhered to the bottom of the plate during the electrical stimulation protocol. During differentiation experiments, media was changed 24 hours after cell seeding to a differentiation media, namely, DMEM with 5% Horse Serum (HS) and 1% Am/Ab (to be referred to as DM) and the electrical stimulation protocol occurred 24 hours post media change. Pharmacologic treatment of cells included the use of compound C and BAPTA. 5 nM and 10 nM compound C (6-[2-Piperidin-1-yl-ethoxy)-3-pyridin-4-yl-pyrrazolo[1, 5-a]-pyrimidine, Dorsomorphin) was used to inhibit the AMPK pathway and DMSO was added to non-compound C treated cells as a control. 25 μM BAPTA (1, 2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid) was used as a calcium chelator and DMSO again was used as a control.

Electrical stimulation

A Harvard Apparatus Stimulator CS System was used in conjunction with 6-well stimulator plates adapted from Connor et al [1] The stimulator parameters were based on previous optimization experiments as follows Cells were stimulated for 4 hours/day (voltage at 10V, frequency at 5 Hz, width at 5ms, delay of 2 ms and the system was set to an alternating mode in order to change the direction of the current) at 37°C at 5% CO_2 followed by a 20 hour recovery period, 24 hours after the plating of the cells An optimization experiment was run to determine the optimal number of consecutive days for electrical stimulation to occur This experiment tested 1 through 5 days of electrical stimulation Media changes occurred at 3 days electrical stimulation to ensure proper nutrient availability for the cells throughout the experiment

Cell Harvesting

Cells were harvested 20 hours post treatment Wells were aspirated to remove media, and subsequently washed twice with cold Phosphate Buffered Saline (PBS) Cells were collected using a 25 cm cell scraper and transferred into Eppendorf tubes along with 1 mL of cold PBS Cells were then centrifuged at 5000rpm and 4°C for 10 minutes Supernatant was discarded and the pellet was resuspended in TENT ++(0 2% Tent [TRIS, EDTA, NaCl and 0 2% Triton x-100], Sigma protease inhibitor cocktail, Sigma phosphatase inhibitor cocktail 2)

Samples were then sonicated for 3 seconds, and centrifuged for 10 minutes at 13, 200 rpm The supernatant lysate was then extracted and stored at -84°C

Immunoblotting

Sample protein concentrations were determined by Bradford Assays 12 5-25 µg of protein was loaded onto 12% SDS PAGE gels and proteins were separated at 120V for 90-120 minutes and transferred to a PVDF membrane overnight at 20V Blots were amido black stained to evaluate protein loading and transfer Membranes were blocked for 2 hours in 10% low fat milk Blots were incubated in primary antibodies for $p27^{Kip1}$[1 5000], p27-T198[1 2000], GAPDH[1 10,000], p-AMPK[1 1000], AMPK[1 1000], Cyclin E[1 1000], MyoD[1 1000] and COX IV[1 1000] overnight at 4°C Following incubation, the membranes were washed with a saline detergent (Tris-Buffered Saline with 0 5% Tween, TBST) Secondary antibodies [1 5000] were incubated with the membrane at room temperature in 5% milk for 1 hour A Chemiluminescent HRP Substrate was used to detect secondary antibodies on the membranes All experimental samples were run on the same membrane to avoid variability The Kodak In vivo FX Pro Imager was used to detect the chemiluminescence signal Signals were quantified using Carestream molecular imaging software

Immunoprecipitation

An immunoprecipitation assay was utilized to test the amount of Cyclin E bound to p27 within samples 100 µg of protein per sample underwent a pre-

clear stage (anti-rabbit side chain [1:2000]) before being incubated with primary p27 antibody for 1 hour at 4°C. Protein A-Sepharose beads were added to the lysate/antibody mixture and incubated for 1 hour at 4°C. The precipitated proteins were dissociated and immunoblotted according to the already stated *Immunoblotting* methodology.

Statistical Analysis

A one-way ANOVA was used to analyze the effect of electrical stimulation on various protein levels in cells cultured in both PM and DM C2C12 myoblasts. A regression analysis was completed on free cyclin E protein content over days of electrical stimulation. A correlation analysis was completed on the relationship of various proteins measured. A two-way ANOVA was used to test the effects of electrical stimulation and Compound C treatment on various protein levels. These experiments are n=3-5. No statistical analyses were run on the BAPTA treated cells as this experiment only has 2 replicates (n=2). Statistical analyses of treatments that give $p \leq 0.05$ will be considered significantly different. Data are GAPDH corrected (loading control) as well as corrected for control cells (that is, day 3 protein values are the result of dividing day 3 stimulated value by day 3 control value).

RESULTS

Electrical stimulation causes cell cycle arrest in C2C12 myoblasts

Initial experiments were designed to determine the effect, if any, electrical stimulation had on C2C12 myoblast cells cycling status. Upon visual inspection of cells as days of electrical stimulation increased, there were apparent differences between control cells and stimulated cells in both PM and DM. In PM cells the control cells represent a typical growth progression through 0 to 3 days. The stimulated PM cells demonstrated far fewer cells by day 3, despite beginning with similar cell number on day 0 as the control cells (Figure 6). This result is indicative of cell cycle progression being slowed. Within DM cells, the control cells represent typical growth patterns. The DM control cells appear to have a typical swirling appearance by day 3. In the stimulated cells on the other hand, no swirling occurred, however premature and atypical myotube formation occurred. Additionally, there were apparent differences in the appearance of the cells cultured in PM (A-F) versus DM (G-L) as cells in DM begin the differentiation process more rapidly. Figure 6 shows a picture of the cells at various stages of electrical stimulation magnified by 10X.

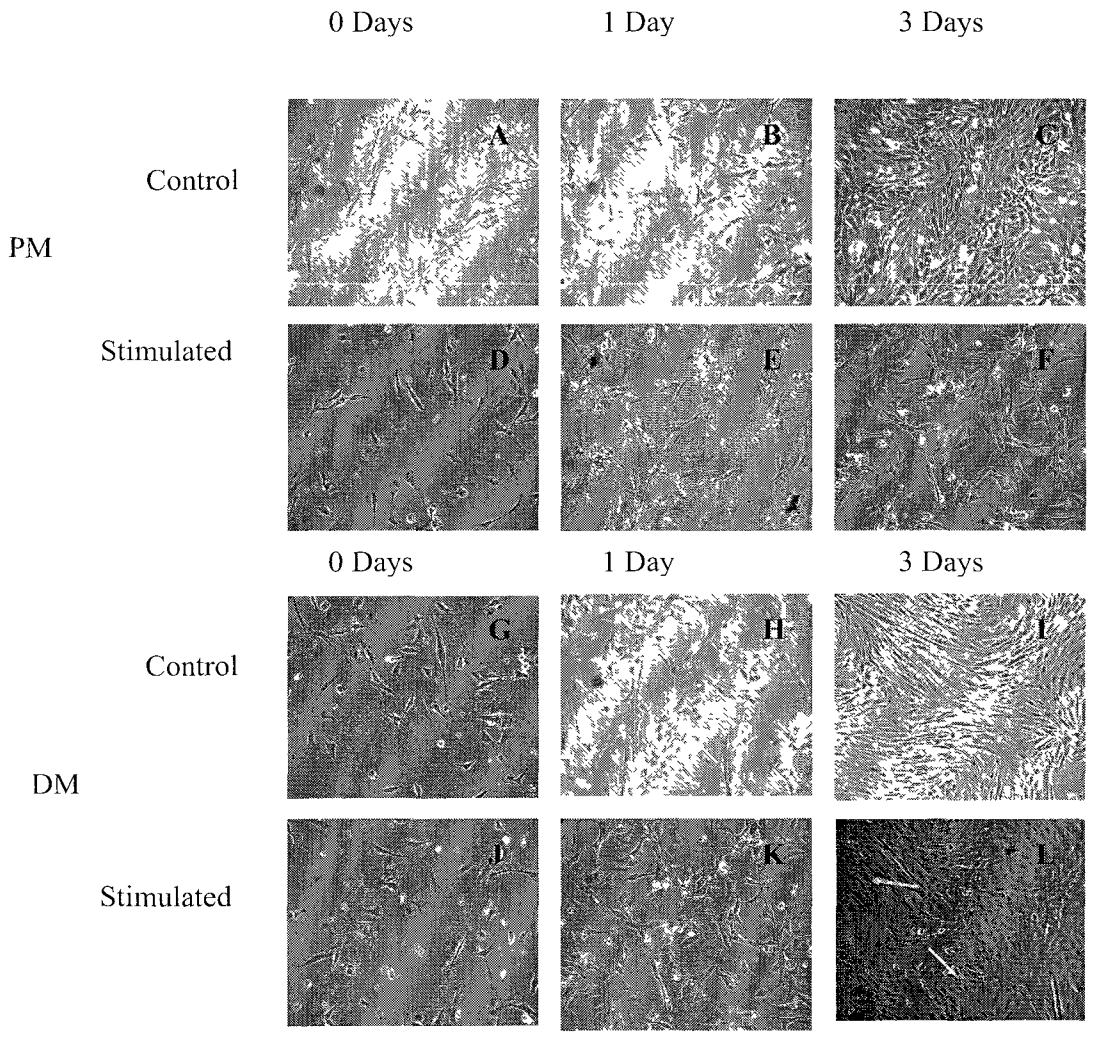

Figure 6. Microscopic pictures of C2C12 myoblasts in both PM (A-F) and DM (G-L) cells. A, D, G, and J represent the cells at day 0 of treatment. B, E, H, and K represent cells at day 1 of treatment. Finally, C, F, I, and L represent cells at day 3 of treatment. Additionally, A, B, C, G, H, and I represent control cells. D, E, F, J, K, and L represent stimulated cells. The arrows present in L show premature (compared to control [I]) myotube formation.

The protein expression of p27, cyclin E as well as free cyclin E levels (determined by immunoprecipitation) of cells subjected to both PM and DM can be found in Figure 7 and 8. p27 protein content significantly increased over days of electrical stimulation in cells cultured in both PM and DM (Figure 7A,B and 7A,C, respectively). p27 protein expression was more drastically increased in PM cells reaching a maximum at day 5 of over 5 fold above control (Figure 7B). p27 protein levels in DM cells reached a maximum at 5 days as well, however only reached levels just over 2 fold of control (Figure 7C). This dramatic increase in p27 protein expression indicates that cell cycle inhibition is occurring.

Cyclin E protein expression in both PM and DM cells significantly decreased by day 5 of electrical stimulation (Figure 8A-C). By day 5 of electrical stimulation levels significantly dropped to over 50% of those observed in day 2 cells. Cyclin E levels in DM cells experienced a greater depression than seen in PM cells. In DM cells, cyclin E protein content reached significantly lower levels by day three and reached lowest levels by day 5 of electrical stimulation reaching 25% of those seen in non-stimulated cells. These results taken in concert with the protein expression levels of p27 indicate a cell cycle inhibition. However, regardless of the total levels of cyclin E, the true indicator of the cells drive to progress through the cell cycle are the levels of free cyclin E, that is, cyclin E which has not been bound by its CKI p27.

Figure 8D and 8E respectively show free cyclin E levels present in PM and DM cells. To calculate free cyclin E content, cyclin E levels measured via the immunoprecipitation were corrected for p27 levels measured (as seen in Figure 17). Next a ratio of bound cyclin E (I.P. Western Blot) to total cyclin E. When this ratio of bound cyclin E: total cyclin E is inverted, it will produce free cyclin E protein levels. The slope in both the PM and DM cells are similar in magnitude and demonstrate a significant reduction in free cyclin E over days of electrical stimulation. These data allowed for a more complete interpretation of changes in total protein and points to the physiological significance of the cyclin E protein content decreases seen in Figure 8B and 8C.

Overall, the significant increases in p27, decreases in total cyclin E, and decreases in free cyclin E indicate an apparent "turning off" of the cell cycle. The protein quantification of these cell cycle regulators match the microscopic images discussed previously (Figure 6).

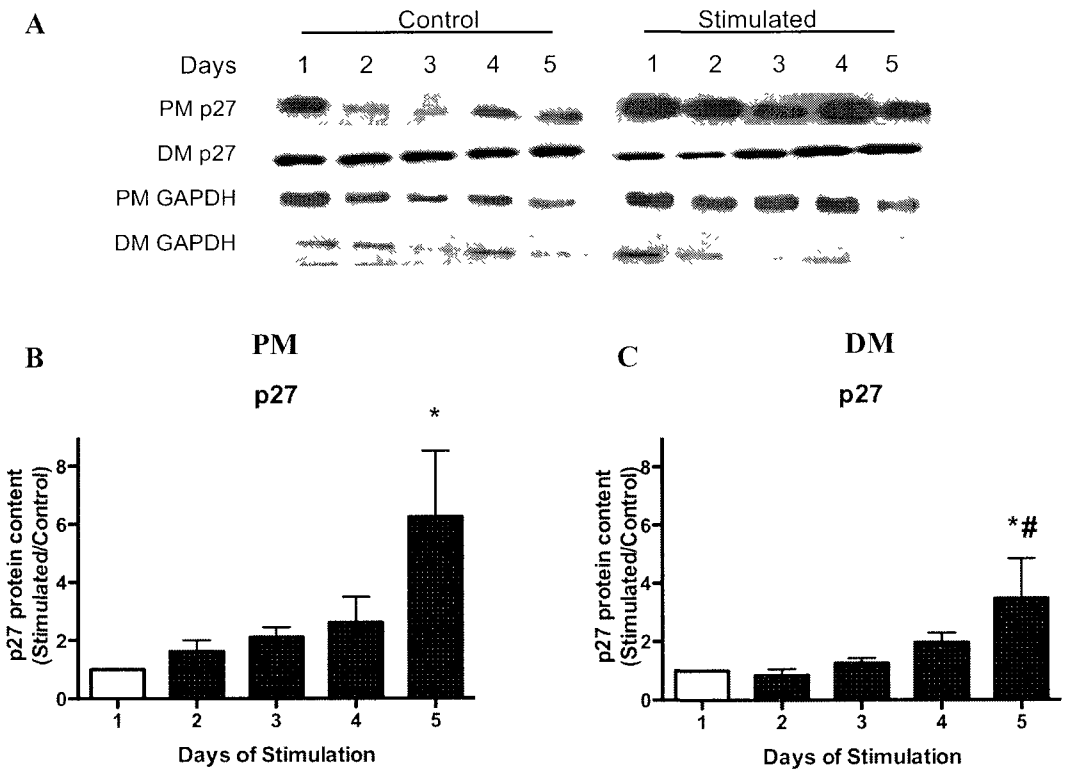

Figure 7. Protein expression of p27 in cells exposed to both PM (B) and DM (C). p27 protein changes were greater in PM (reaching a 5 fold increase) compared to DM (reaching 2 fold). * represents a statistically significant difference from control; # represents a statistically significant difference from day 2 of electrical stimulation.

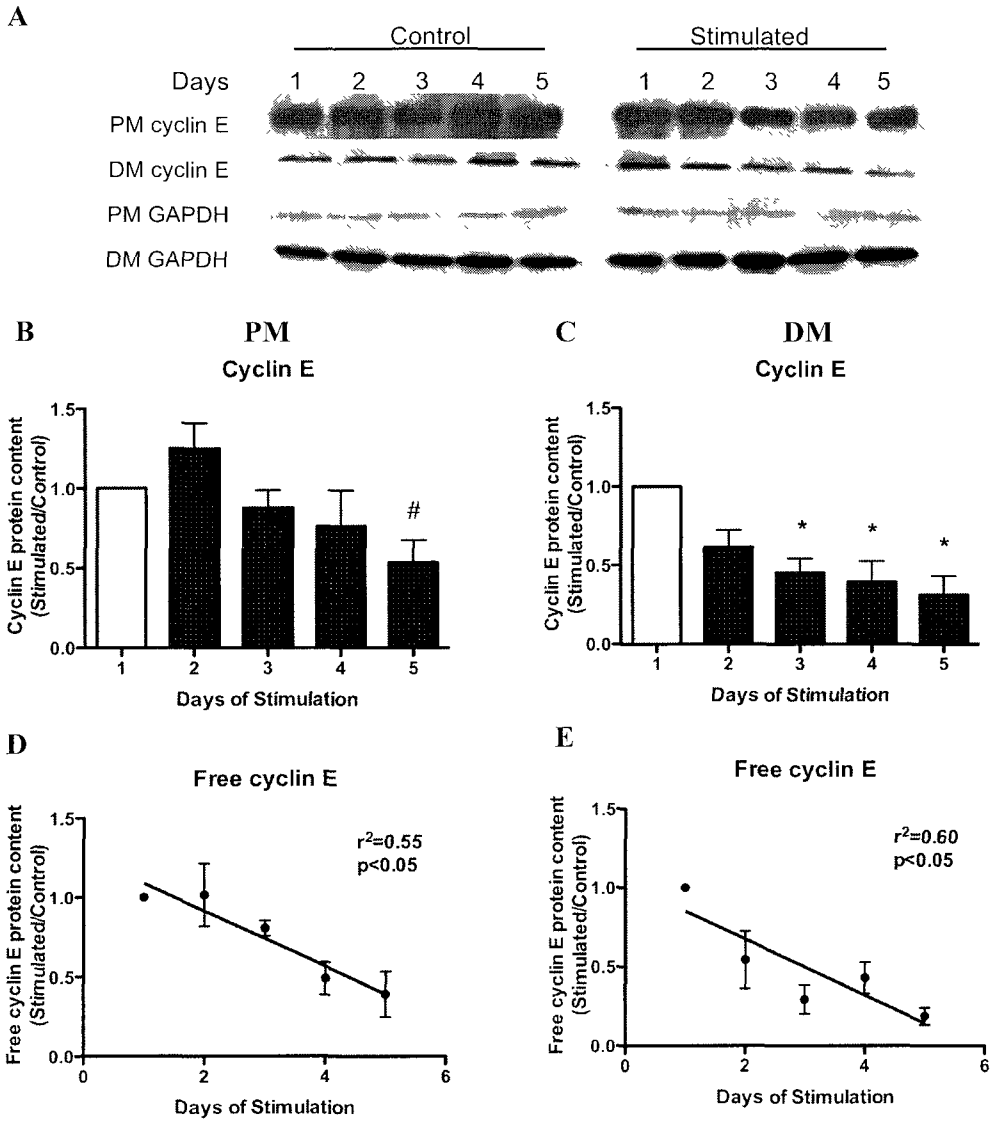

Figure 8. Protein expression of cyclin E (A, B, and C) and free cyclin E (D, E) exposed to both PM (B, D) and DM (C, E). Cyclin E levels of DM cells responded more drastically to electrical stimulation treatment than those of PM cells, however free cyclin E levels of both PM and DM cells appeared to have responded similarly. * represents a statistically significant difference from control; # represents a statistically significant difference from day 2 of electrical stimulation.

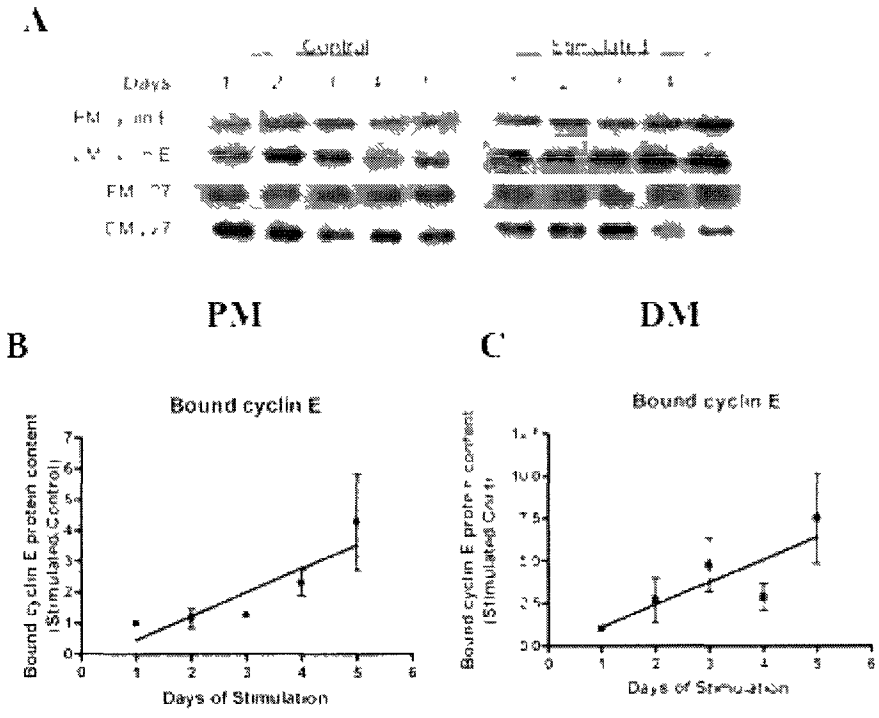

Figure 9. Protein expression of bound cyclin E in PM and DM cells Both PM (B) and DM (C) cells experienced increases in bound cyclin E over days of stimulation These data were corrected for total cyclin E levels and subsequently inversed to determine free cyclin E levels seen in Figure 8

AMPK as a regulator of electrical stimulation induced cell cycle alterations

It is clear from the results observed in the previous section that electrical stimulation does in fact have an effect on cell cycle proteins in C2C12 cells in both PM and DM conditions. There must be an upstream pathway which is regulating these changes in protein expression AMPK was investigated as a possible target for regulating these changes since AMPK targets phosphorylation of the T198 residue on p27 and AMPK has been implicated previously to rise in whole fibre studies due to electrical stimulation. Phosphorylated AMPK (pAMPK; on Thr 172) protein content was measured to investigate whether the electrical stimulation was having an effect on this pathway Figure 10 illustrates the pAMPK (A, B, C) and total AMPK (A, D, E) protein levels over days of electrical stimulation In PM cells, pAMPK protein levels reached significant increases by day 3 of electrical stimulation, peaking at approximately a doubling compared to control Total AMPK levels in the PM cells were unchanged at all time points In DM cells, pAMPK levels also reached a statistically significant increase at day 3 of electrical stimulation, however in contrast to PM cells, the DM levels reached a maximum of almost a 4 fold increase compared to control Although day 3 was the only statistical significant increase found within these data, it is noteworthy that day 2, 4, and 5 levels were all at least twice as high as day 1 levels (same increase as seen in PM cells) A 2 fold increase in pAMPK may represent

more of a physiological significance than the statistical tests imply. Again total AMPK levels in the DM cells remained unchanged. The electrical stimulation induced increase in pAMPK indicates that AMPK may be activated by electrical stimulation and involved with regulating the cell cycle protein changes observed earlier.

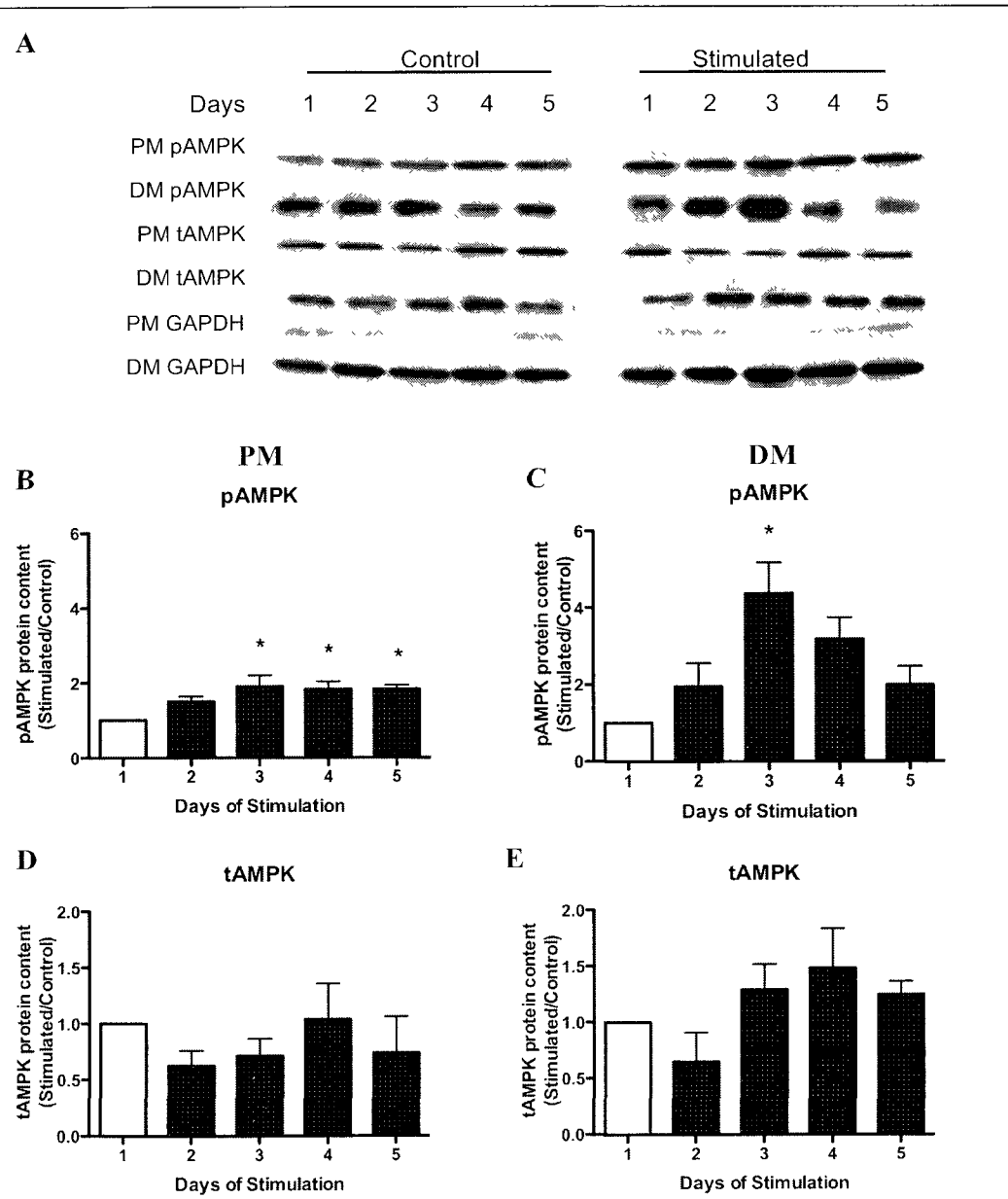

Figure 10. Protein expression of pAMPK (B, C) and tAMPK (D, E) in PM (B, D) and DM (C, E) cells. In both PM and DM cells, pAMPK levels were significantly increased with days of electrical stimulation. PM cells doubled in pAMPK levels, where as the DM cells reached nearly a 4 fold increase in pAMPK expression. Conversely, tAMPK levels remained unchanged in both PM and DM cells. * represents a statistically significant difference from control.

As previously stated, activated AMPK has a phosphorylation target residue on the p27 protein, namely T198. Due to the fact that p27 levels showed an increase over days of electrical stimulation, and AMPK was also increased due to electrical stimulation, I decided to measure the protein expression of p27 which has been phosphorylated on the T198 residue. These data will indicate whether the increase in pAMPK seen is then acting on p27 by phosphorylating it at T198 and stabilizing the protein. Figure 11 gives the T198-p27 expression profiles over days of electrical stimulation in both PM (B) and DM (C) cells. In PM cells, T198-p27 was significantly increased by day 5 of electrical stimulation reaching a maximum of approximately 2 fold above control. These values correspond to the pAMPK protein rise observed in the PM cells. T198-p27 protein content was also significantly increased in the DM cells, although to a much greater extent. By day 4 of electrical stimulation T198-p27 levels in the DM cells reached statistical significance and reached a maximum at day 5 of approximately 7 fold compared to control. Interestingly, the pAMPK protein levels of the DM cells were more drastically increased compared to PM cells as well. It seems as though the drastic increase in pAMPK of the DM cells lead to a correspondingly larger increase in T198-p27 protein content compared to PM cells.

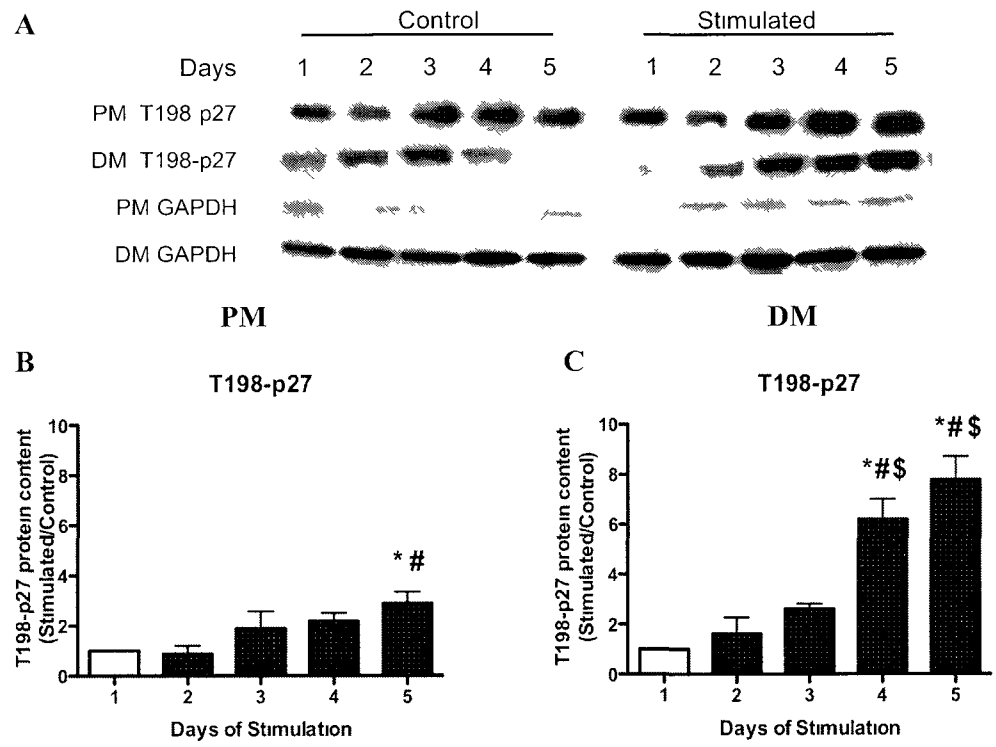

Figure 11. Protein expression of T198 p27 in PM and DM cells T198 p27 was significantly increased over days of electrical stimulation although DM cells reached much higher levels PM cells reached a maximum approximately a 2 fold increase compared to control at 5 days of electrical stimulation DM also reached a maximum increase at 5 days of electrical stimulation, however achieved almost an 7 fold increase compared to control * represents a statistically significant difference from control # represents a statistically significant difference from day 2 of electrical stimulation $ represents a statistically significant difference from day 3 of electrical stimulation

The pAMPK/T198-p27 pathway is involved in C2C12 cell cycle arrest

It is apparent that there are a variety of cell cycle related events that occur to due electrical stimulation Thus far, promising results have been shown implicating cyclin E, p27, pAMPK and T198-p27 as being involved with electrical stimulation effects on the cell cycle To further investigate this pathway within the electrical stimulation model, correlation analyses were preformed on the existing data (Figure 12) T198-p27 protein levels were correlated with total p27 levels to demonstrate the stabilizing effect of this particular phosphorylation (Figure 12 A and B) In both PM and DM cells, significant correlations were found When T198-p27 levels rose, p27 levels did as well indicating a stabilizing effect at this phosphorylation on this protein Next, a correlation was completed on both PM and DM cells for T198-27 and free cyclin E (Figure 12 C and D) The intention of this test was to analyze whether the decreases in free cyclin E due to electrical stimulation are related to the increases in T198-p27 The correlation was significant in both PM and DM cells These data suggest that as levels of T198-p27 increase, levels of free cyclin E decrease The final correlation conducted investigated the relationship of pAMPK and free cyclin E (Figure 12 E and F) Again, in both PM and DM cells, as pAMPK levels rose, free cyclin E levels decreased These significant correlations demonstrate the importance and relevance of the pAMPK/T198-p27 relationship in decreasing free cyclin E levels thus leading to cell cycle arrest

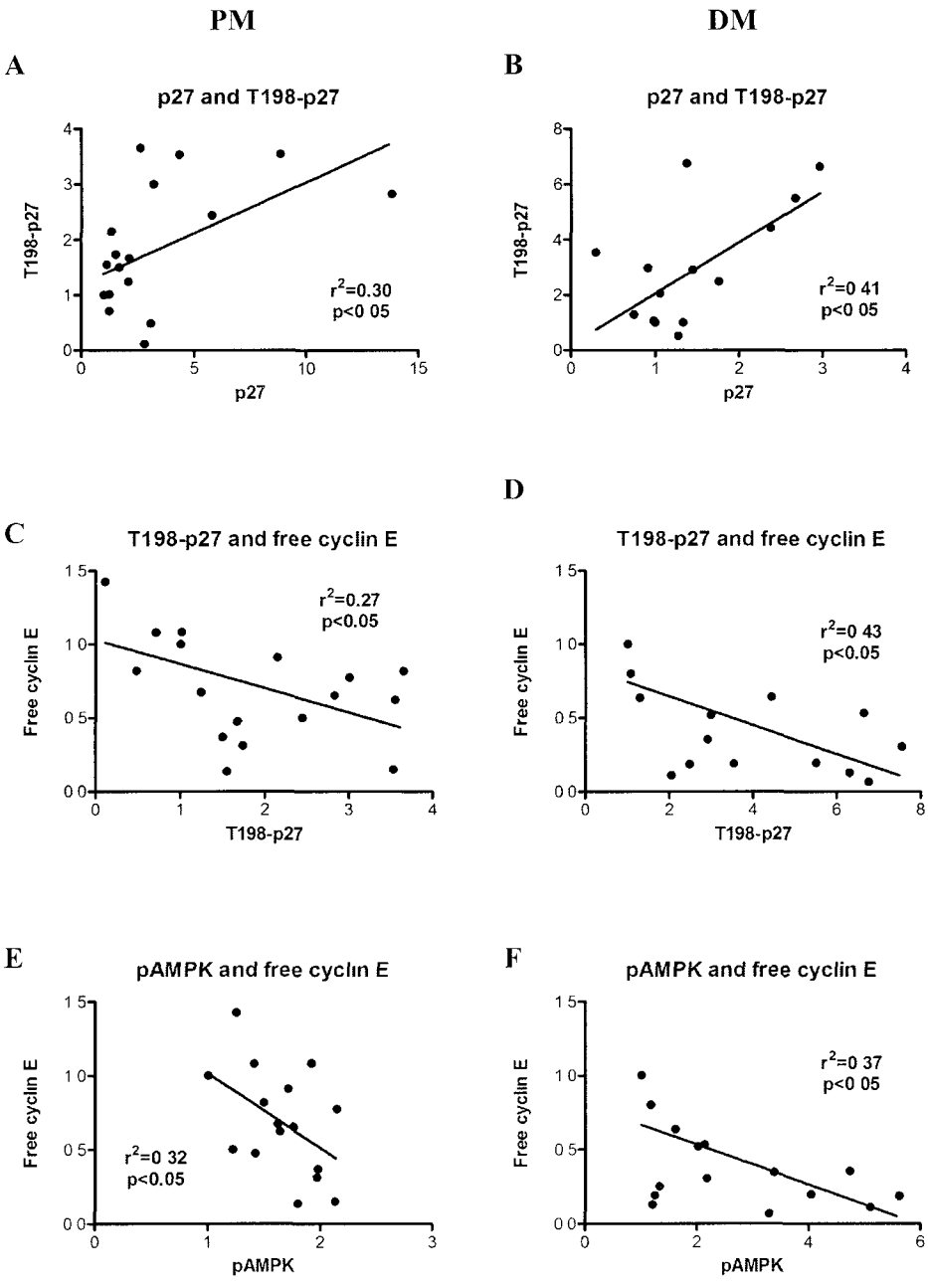

Figure 12. Correlation data preformed on existing data All correlations are statistically significant A, C, and E are proliferating media cells, where B, D, and F are differentiating media cells

AMPK is pivotal for electrical stimulation induced cell cycle arrest

As AMPK has now been implicated in moderating the cell cycle arrest seen in both PM and DM cells due to electrical stimulation, compound C was used to inhibit the activation of AMPK. This experiment required a substantial optimization experiment to determine the ideal concentration of compound C to use to treat the cells. Initially, a 10 nM treatment of compound C was used, however by day 3 of treatment the cells did not appear to be healthy and were dislodging from the bottom of the plate. The optimization experiment completed for various compound C concentrations is outlined in Appendix 1A. Seen in the microscopic picture of C2C12 myoblasts after 3 days of compound C treatment of 5 nM, it is clear that these cells are viable and healthy (Appendix 1B). Finally pAMPK protein was quantified for each sample and it is clear that the 5 nM compound C treatment provided the greatest magnitude of AMPK inhibition (Appendix 1C). This level of pAMPK inhibition seen with the viability of the cells seen in Appendix 1B was enough evidence to switch the treatment concentration of compound C from 10 nM to 5 nM.

Inhibiting activation of AMPK while subjecting the cells to electrical stimulation will determine whether AMPK phosphorylation is necessary for downstream cell cycle arrest to occur. Figure 13 simply shows the effectiveness of compound C in inhibiting AMPK phosphorylation. In PM cells significant inhibition of pAMPK was observed at day 2 and day 3 of electrical stimulation (Figure 13 A,B). In DM however, a significant inhibition of pAMPK was

only achieved at day 3 of electrical stimulation (Figure 13 A,C). No changes in total AMPK protein content was observed in PM and DM cells with electrical stimulation or compound C treatmeant in conjunction with or separate from each other (Figure 13 D and E). Significant differences in T198-p27 were found however in both PM and DM cells (Figure 14). Both PM and DM cells treated with DMSO increased T198-p27 protein levels in cells exposed to electrical stimulation as previously observed. When treated with compound C over days of electrical stimulation, the T198-p27 increases saw in the DMSO cells were radically decreased. PM cells observed a significant decrease in T198-p27 phosphorylation with compound C treatment at day 2, whereas DM cells saw a significant decrease at day 3. When subjected to electrical stimulation from 1 through 3 days, cells treated with DMSO had typical electrical stimulation responses as seen before (Figure 7). That is, p27 levels increased, whereas cyclin E levels decreased. In both PM and DM cells treated with compound C, p27 levels were significantly decreased (Figure 15 B and C, respectively) compared to their DMSO controls. In PM cells there appears to be no trend within the compound C treated cells over day of electrical stimulation, unlike the significant increases seen in the DMSO treated cells. In DM cells however, increases in p27 levels do occur in the compound C treated cells, however are drastically lower than DMSO treated cells. Protein content of cyclin E decreased with days of electrical stimulation in DMSO treated cells in both PM and DM. In proliferating media cells treated with compound C, protein levels of cyclin E

increased with days of electrical stimulation. Initially at day 1, DMSO treated cells expressed higher amounts of cyclin E, however by day 3 of electrical stimulation, compound C treated cells expressed more than double the amount of cyclin E than the DMSO treated cells. Furthermore, in DM cells similar results were observed. At day 1 of electrical stimulation compound C treated cells expressed 50% of the cyclin E expressed in DMSO treated cells, however by day 3 of electrical stimulation the compound C treated cells expressed almost double the amount expressed in DMSO treated cells. This is strong evidence implicating AMPK as a pivotal mediator regulating the expression of p27 and cyclin E resulting in cell cycle arrest of C2C12 myoblasts due to electrical stimulation.

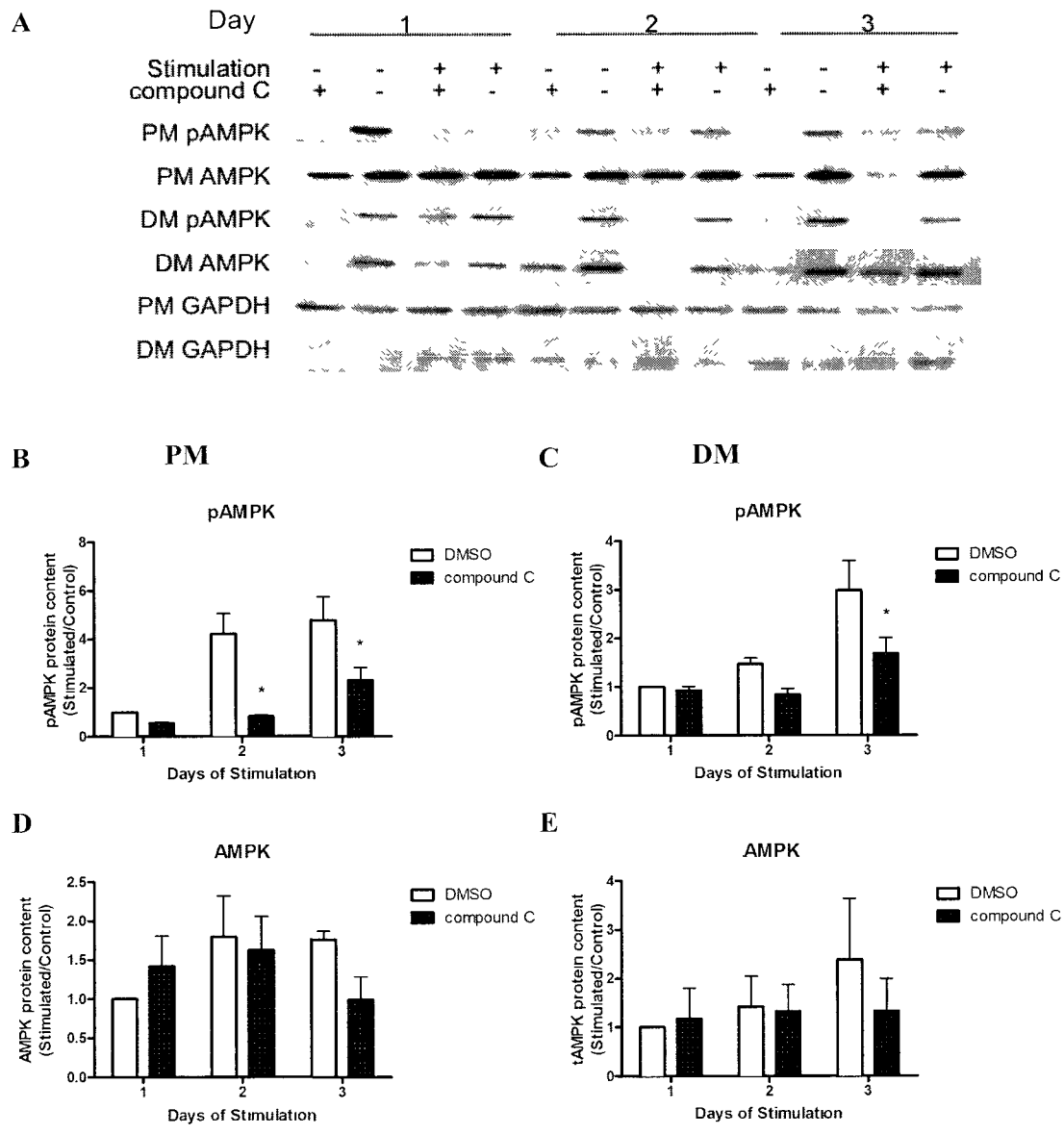

Figure 13. Protein expression of pAMPK (A, B, C) and tAMPK (A, D, E) in PM (B, D) and DM (C, E) cells. Cells treated with compound C showed significantly decreased pAMPK expression when compared to same day controls. There were no statistical significances in total AMPK levels over days of electrical stimulation or with compound C treatment. * represents a statistically significant difference compared to same day control (DMSO treated

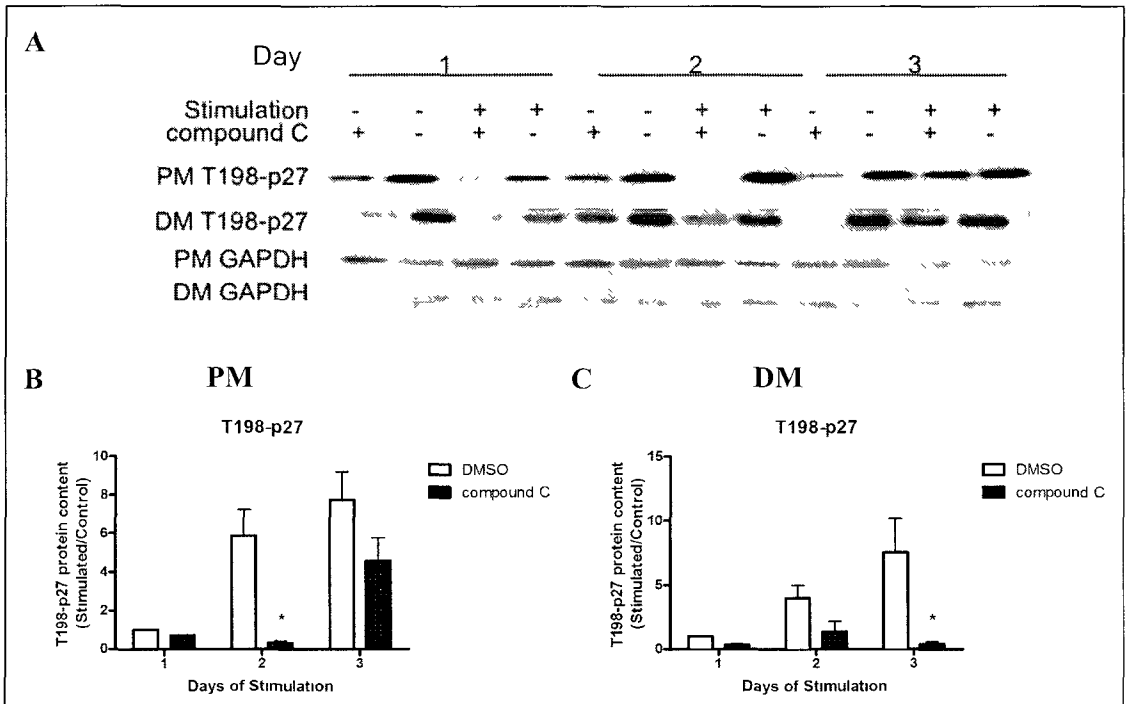

Figure 14. Protein expression of T198-p27 in PM and DM cells over days of electrical stimulation treated with either DMSO or compound C. Cells treated with DMSO experienced increases over days of electrical stimulation, whereas cell treated with compound C did not. * represents a statistically significant difference from control.

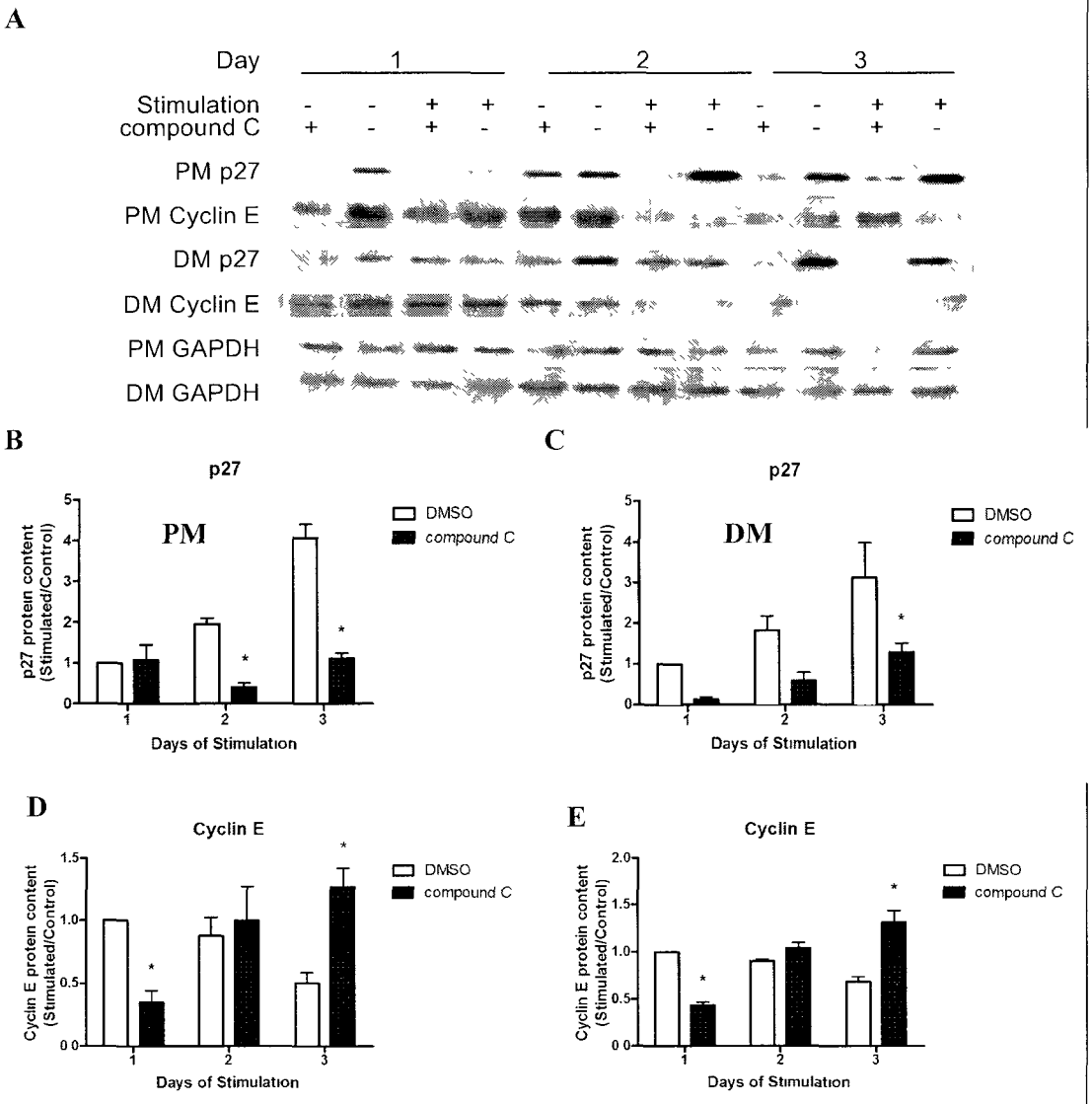

Figure 15. Protein expression of p27 and cyclin E treated with compound C or DMSO (control) over days of electrical stimulation. Protein expression of p27 in both PM (B) and DM (C) cells treated with compound C were significantly lower than the cells treated with DMSO. The protein expression of cyclin E in both PM (D) and DM (E) cells treated with compound C were significantly different than cells treated with DMSO. * represents a statistically significant difference compared to same day control (DMSO treated cells).

Cell cycle arrest due to electrical stimulation may increase the rate of myoblast differentiation

Initially, to investigate the differentiation effects of the electrical stimulation induced cell cycle arrest COX IV was utilized as an indirect marker of myoblast differentiation status. As previously described, COX IV protein expression has been found to increase with days of differentiation of C2C12 cells [1]. Figure 16 shows COX IV expression in both PM and DM conditions. In PM cells, levels of COX IV protein increased with days of electrical stimulation, reaching a maximum of almost 2 fold over control. By day 5 of electrical stimulation however, COX IV levels fell back to day 1 levels. In DM cells however, there were no observed changes in COX IV protein expression over days of electrical stimulation. This is indirectly indicative that in PM cells electrical stimulation may increase the differentiation status in myoblasts. Furthermore, MyoD protein levels were measured in both PM and DM conditions to asses more directly the differentiation status of the cells (Figure 17). In PM cells, MyoD protein levels were significantly increased by day 3 of electrical stimulation reaching a maximum of almost 4 fold over control. Interestingly, the MyoD profile for PM cells follow a similar trend to that COX IV protein levels. DM cells also saw significant increases in MyoD protein by day 3 of electrical stimulation and reached a maximum of 2 fold above control. Taken together, the COX IV and MyoD results are indicative of an

advancing differentiation status in both PM and DM cells due to electrical stimulation.

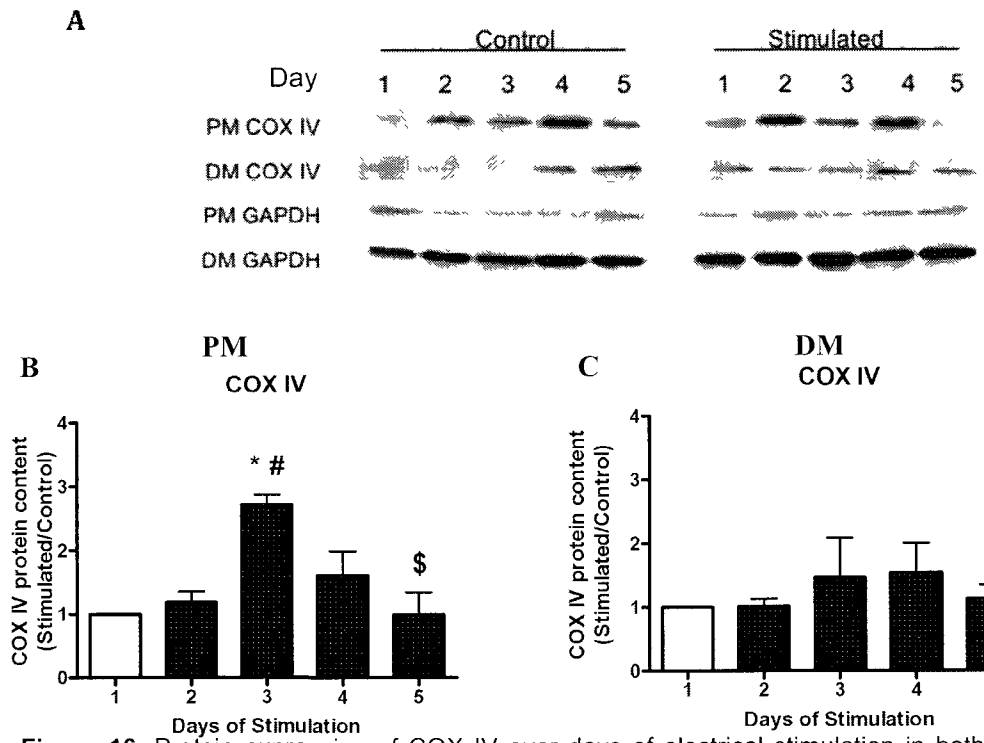

Figure 16. Protein expression of COX IV over days of electrical stimulation in both PM and DM cells. In PM cells statistical significance was reached by 3 days of electrical stimulation from control as well as from day 2. In DM cells no statistical significances were found. * represents a statistically significant difference from control; # represents a statistically significant difference from day 2; $ represents a statistically significant

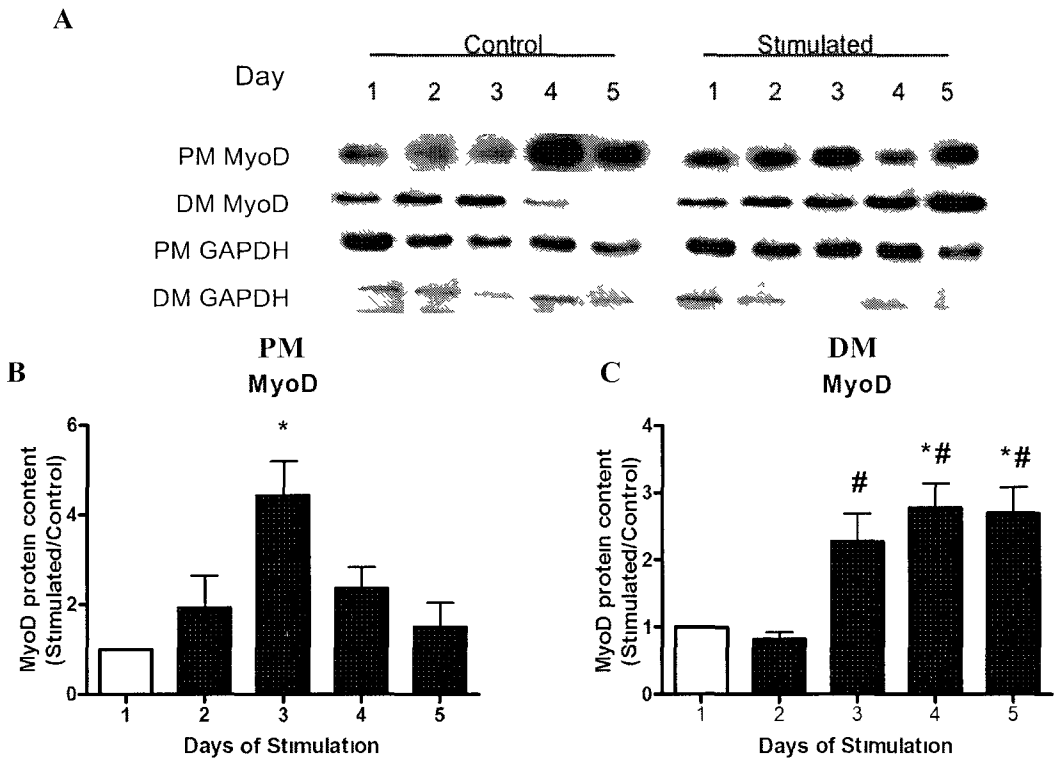

Figure 17. *Protein expression of MyoD over days of electrical stimulation* PM cells (B) saw significant increases, reaching a maximum at day 3 of almost 4 fold over day 1 DM cells (C) saw significant increased from day 2 beginning at day 3 and reaching a maximum of almost 2 fold compared to day 1 * represents a statistically significant difference from control # represents a statistically significant difference from day 2

Calcium may be mediating electrical stimulation dependent AMPK activation in myoblasts

Although the previous data outline the molecular signalling cascade in which electrical stimulation acts to inhibit the myoblast cell cycle, the mechanism in which AMPK becomes activated to mediate cell cycle inhibition is yet to be elucidated AMPK can be activated via multiple avenues, however calcium as a target for AMPK phosphorylation due to electrical stimulation seemed most likely as myoblast cells do not contract and thus do not experience ATP cycling, thus an increased AMP·ATP ratio resulting in AMPK phosphorylation is unlikely in response to electrical stimulation This lead to a pilot experiment investigating calcium's role in this signalling cascade C2C12 myoblasts in proliferating media were treated alone or in conjunction with BAPTA (a calcium chelator) These experiments were run twice (n=2), thus no statistical analyses were completed, however there are clearly evident emerging trends within the data Figure 18 shows p27 and cyclin E protein levels of these treatment conditions at 1 and 2 days p27 levels (Figure 18 B) did not appear to change much at day 1 of treatment, however by day 2 of treatment, electrical stimulation alone elevated p27 levels 700% over day 1 non stimulated cells When these cells were treated with BAPTA in concert with electrical stimulation p27 levels only rose 300% over non-stimulated control cells When calcium is chelated, the electrical stimulation effects seen in p27 are drastically reduced Somewhat disappointing, cyclin E levels did not respond to BAPTA treatment (Figure 18 C)

To further investigate the effects of calcium chelation on this signalling mechanism T198-p27, pAMPK and AMPK levels were measured. Figure 18 shows the data obtained from 2 experiments completed. T198-p27 protein expression mimicked the p27 protein expression trend (Figure 19 B). At day 2, cells treated with BAPTA and exposed to electrical stimulation had a blunted T198-p27 response compared to non BAPTA treated, stimulated cells. In fact, when calcium was chelated, T198-p27 protein levels in stimulated cells were half that of DMSO treated cells. pAMPK response was very similar to both T198-p27 and p27 levels (Figure 19 C). BAPTA treatment blunted the electrical stimulation induced increases seen in pAMPK while total AMPK levels appear to be unchanged over various treatments. The blunted pAMPK expression provides some evidence that in the electrical stimulation induced cell cycle arrest seen in C2C12 myoblasts, calcium is likely the major source of AMPK activation.

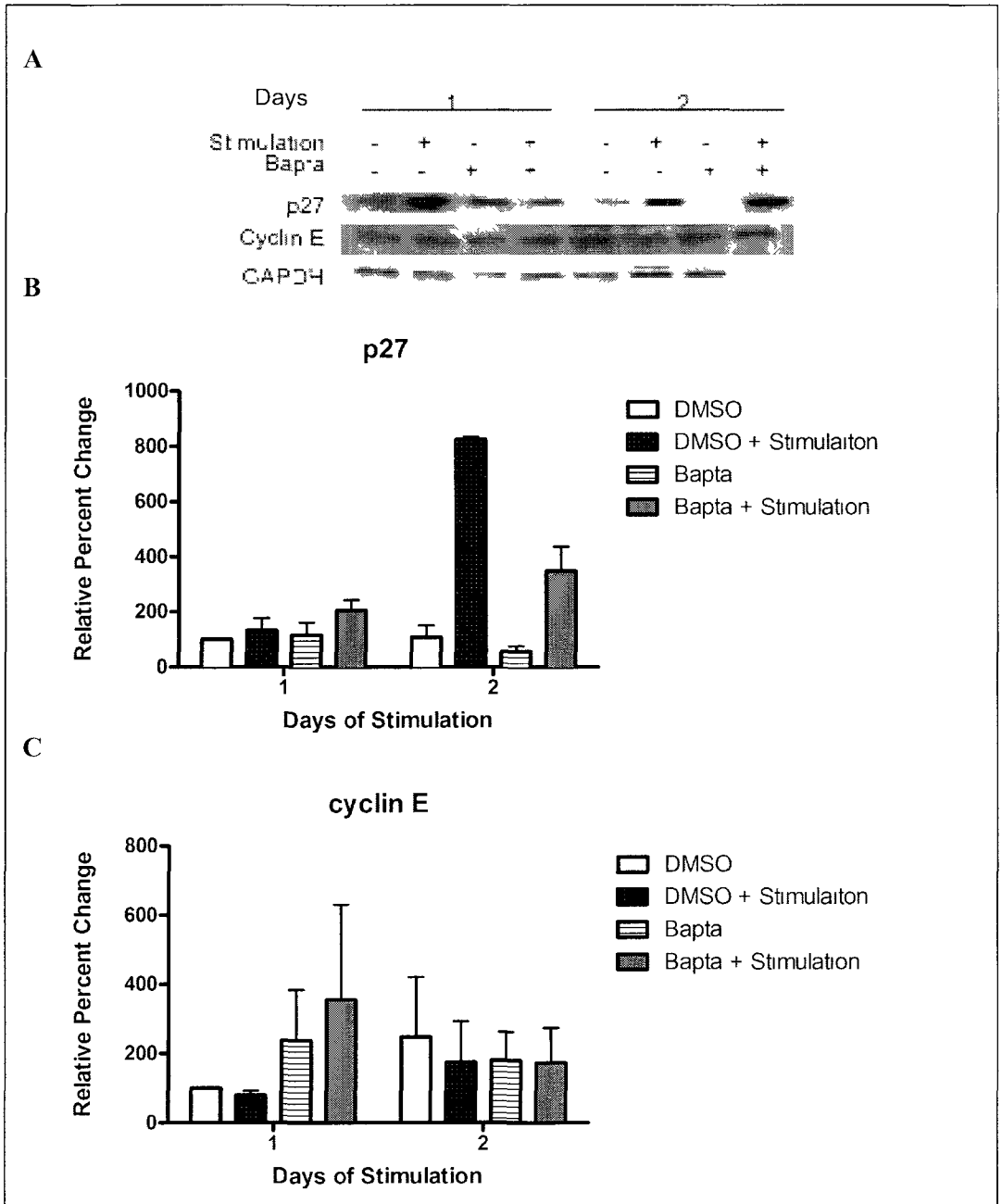

Figure 18. Protein content of p27 and cyclin E in PM in response to electrical stimulation and BAPTA treatment over 1 and 2 days These data represent n=2, thus no statistical analyses were completed

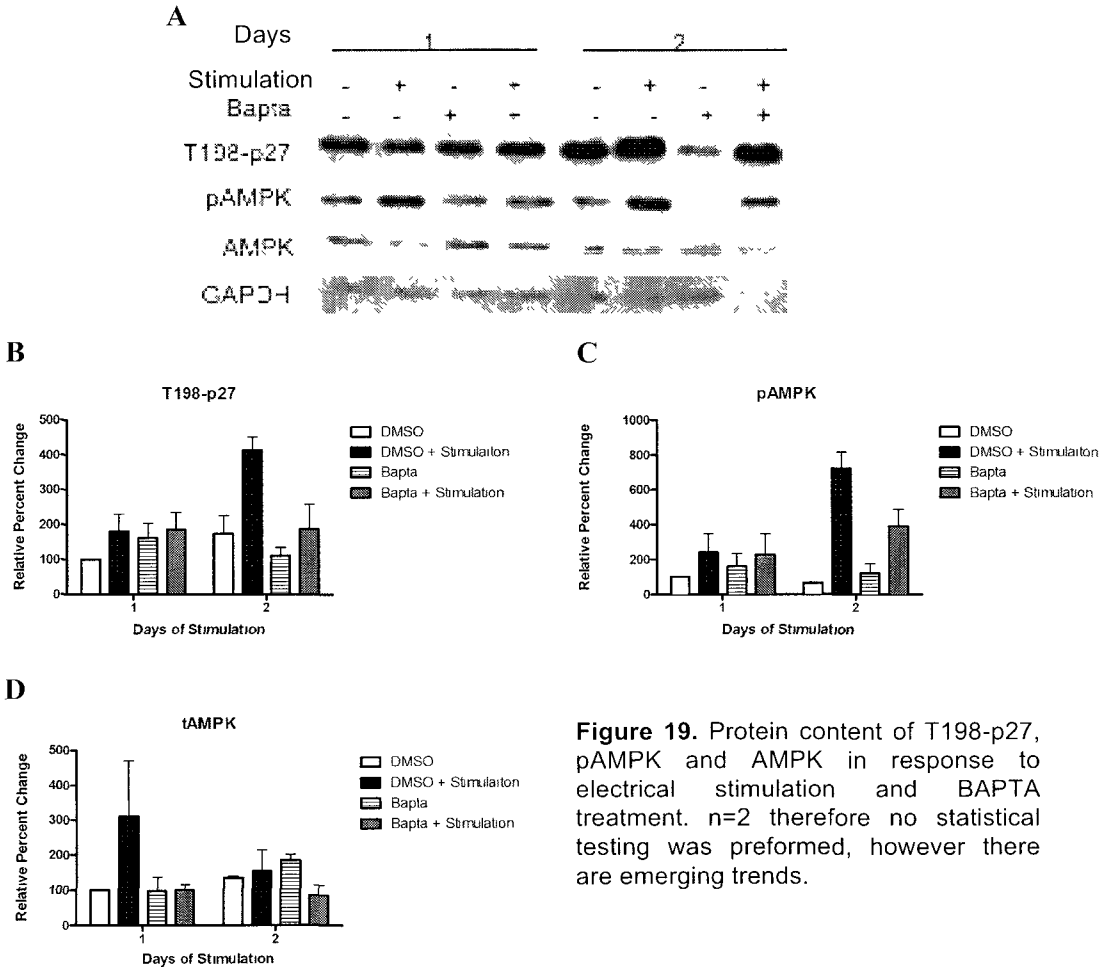

Figure 19. Protein content of T198-p27, pAMPK and AMPK in response to electrical stimulation and BAPTA treatment. n=2 therefore no statistical testing was preformed, however there are emerging trends.

DISCUSSION

Understanding the mechanisms underlying muscle differentiation is crucial for improving muscle related disease as well as muscle injury. There are many factors which can influence the rate of muscle differentiation and many factors which are necessary for differentiation to occur. Furthermore, understanding how the rate of muscle differentiation responds to contraction (exercise) may provide insight to optimal therapy conditions to promote muscle healing in response to injury. As previously mentioned a prerequisite for muscle differentiation is cell cycle cessation and its cell cycle status can be regulated and altered in a variety of ways. To investigate the effects of electrical activation on myoblast cell cycle status and progression through differentiation, C2C12 myoblasts were subjected to electrical stimulation protocols and various cell cycle and myogenic proteins were investigated. This project is extremely novel as to my knowledge no study has investigated the effects of electrical stimulation on myoblast cell cycle in any capacity, hence no direct comparisons can be made between my results and others previously published. Electrical stimulation effects have been investigated with respect to phenotype changes in muscle tissue, however any insight into cell cycle effects have been neglected to date.

Electrical stimulation induces cell cycle arrest in C2C12 myoblasts

Electrical stimulation treatment over days appears to cause cell cycle cessation in C2C12 myoblasts. This was determined based on evidence gathered from experiments which measured increases in p27, decreases in cyclin E and decreases in free cyclin E levels (Figures 7, 8 and 9). The PM cells that were stimulated are drastically less confluent than their control counterparts, which implies cell cycle cessation (Figure 6). These results are obviously not conclusive with regards to cell cycle arrest, although the microscopic pictures are clear. There have been previous reports which show an increase in p27 levels are related to G1 cell cycle arrest [86] therefore I used p27 as an indicator of cycling status in the cells as p27 directly inhibits cyclin E by binding to it and has been recognized as possibly the one of the most important cell cycle inhibitors [68]. The significant increases found in p27 total protein are indicative of cell cycle arrest in many cell lines [87]. The extremely large increases found with electrical stimulation in PM cells (~5 fold) can be explained by the fact that in cycling cells, p27 levels have been shown to be very low [88]. Control cells having such a low basal amount of p27 to begin with, have the capacity for much greater increases due to stimuli than would occur in a population which has higher basal levels. This only strengthens the notion that cell cycle arrest is occurring in these cells. p27 levels are often used as a prognostic marker for various cancers, however if cyclin E levels also rise, there will not be an increased cell cycle arrest.

Cyclin E levels were in fact found to decrease over days of electrical stimulation. These results taken in concert with the rising p27 levels over days of electrical stimulation are a strong indicator of cell cycle arrest. That being said, it may not be absolute and independent quantity of both of these proteins, albeit more importantly may be the ratio of the two or even more telling the amount of cyclin E that is still functional (cyclin E which is not bound by p27). The free cyclin E data (Figure 8) were derived from an immunoprecipitation assay for p27, which was incubated with both p27 and cyclin E antibodies. The end result being a quantified estimate of cyclin E which has been bound by p27. From these data and the previous total cyclin E data, a ratio of bound to total cyclin E can be formed and by inversing these values, free cyclin E profiles are constructed. I believe this is the most telling indicator of cell cycle arrest as it is a measure of functional cyclin E. That is, as this free cyclin E is decreasing, the drive pushing the cell through G1 to S phase is decreasing ultimately leading to arrest. Importantly, the decrease in free cyclin E also provides evidence that the p27 which is being expressed due to electrical stimulation is in fact nuclear as opposed to cytosolic. That is, the additional p27 is entering the nucleus where it binds to cyclin E, resulting in a decrease of free and functional cyclin E. There has been investigation into the relationship between p27 and cyclin E where it was found that p27 and cyclin E have a cooperative relationship with respect to cell proliferation [89]. In another cancer study investigating an anti-cancer compound, G1 phase cell cycle arrest was associated with an increase in

p27 levels and a decrease in cyclin E levels [90]. These studies support the idea that when p27 levels increase and cyclin E levels decrease, cell cycle arrest is occurring, which is the case with my study. The data presented thus far provide strong evidence that electrical stimulation causes G1 cell cycle arrest in C2C12 myoblasts.

Cell cycle arrest due to electrical stimulation may increase the rate of myoblast differentiation

There are apparent differences between protein profiles in PM and DM cells. The observable differences seen between PM and DM cells are likely due to the presence of growth factors in the proliferation media and the absence of growth factors in the differentiation media. DM is often used alone to induce differentiation [91], which explains the more advanced myogenic status of the DM cells. In a study which treated C2C12 myoblasts in DM conditions with adiponectin, inducing differentiation, microscopic pictures were also taken [91]. This study also clearly shows a difference between DM cells and adiponectin treated cells. Specifically, the adiponectin treated myotubes were much smaller than the DM myotubes and were not as organized. The authors report that both DM and adiponectin treatment increase the rate of differentiation, however DM is much more effective. An additional study which treated C2C12 cells with AICAR (AMP mimetic), found that the myotube diameter in AICAR treated cells were significantly decreased compared to control after 24 hours [81]. My results

are consistent with these results, in that electrical stimulation resulted in premature myotube formation, however the cells appeared to be undersized, disorganized and may not be functional This is indicative that electrical stimulation may induce the expression of myogenic proteins prematurely with the cells not entirely prepared for differentiation This may result in the atypical formation of undersized myotubes In the DM cells by day 3 in the control group a swirling of the cells begins to occur which is indicative of the onset of myotube formation In the stimulated cells however, no swirling occurs, instead random and small myotube formation has occurred It is unknown whether these myotubes are functional, albeit they are smaller than what appears in control cells Additionally, because there is no swirling of the cells, the myotube formation is sporadic and appears disorganized These cells however are at a further differentiation point than cells in PM conditions, as PM cells never experienced myotube formation, only cell cycle arrest There have been reports of more successfully increasing the rate of differentiation while maintaining the integrity of the myotubes A study treated cells with Smad7 and found a clear increased rate of differentiation while maintaining the typical and functional appearance of myotubes [92] Aside from apparent visual differences between PM and DM cells, molecular differences in protein expression were also found Although in both PM and DM conditions p27 levels rose, the magnitude to which they rose to was different in each condition p27 levels in PM conditions reached higher levels than in DM conditions The difference in p27 levels in PM and DM conditions has

also been found in previous reports in intestinal epithelium [93] This report found that p27 levels were also highest in proliferating conditions compared to differentiated conditions, which is consistent with my data As the only difference between the PM and DM groups is the presence of growth factors, this is likely the cause of the differences experienced Previous work has shown that p27 levels in cycling cells (proliferation media conditions) is extremely low compared to p27 levels in DM [88] Obviously, starting with a higher content of p27, DM cells will not have the capacity to generate as large increases as PM cells

As with the p27 levels, there were differences between the PM and DM cyclin E profiles PM cells experienced less of a decrease in cyclin E than did DM cells While the decrease in cyclin E in PM conditions is not as large as the decrease in DM cells, p27 levels in PM cells were more elevated than in DM cells Additionally, cyclin E levels in DM cells have previously been found to be much lower than cyclin E present in cycling cells [88] Interestingly, free cyclin E levels in both PM and DM cells were decreased with similar magnitudes, unlike the total p27 and cyclin E protein levels This shows that more important than the absolute change in p27 and cyclin E is the amount of free cyclin E which is available to push the cell through G1 Although there appeared to be differences between PM and DM conditions with both p27 and cyclin E levels, that is higher levels of both proteins in PM conditions, the free cyclin E levels measured provided true insight into the functional similarities between both growth conditions Specifically, regardless of the profile differences in the proteins

between PM and DM conditions it seems as though the electrical stimulation induced arrest is consistent between both conditions

Differences were also evident between with respect to pAMPK as well in PM and DM conditions In DM cells the increases are much greater, however only reach significance at day 3 of electrical stimulation Noteworthy is the fact that although at days 2, 4 and 5 no significance was found, the magnitude of the increases are at least the same as in the PM condition or greater It seems as though the physiological relevance of these data are greater than what the statistical tests may imply The pAMPK levels at day 3 seen in the DM condition are very comparable to pAMPK levels after 3 days of adiponectin treatment In fact, in a study that treats cells with adiponectin for 72 hours in differentiation media, similar to the media used in this study, saw approximately a 5 fold increase in pAMPK With electrical stimulation in my study a 4 fold increase was found at day 3 [91] After day 3 of electrical stimulation, the decreases in pAMPK in the DM condition might be due to the length of time the AMPK protein remains phosphorylated The phosphorylation event on AMPK does not last for a substantial amount of time In contrast, it seems reasonable that the reason for the difference between the pAMPK responses in PM and DM conditions is the growth factors present in the PM condition In differentiation media, the changes in pAMPK due to electrical stimulation will likely be more pronounced as the cells have essentially been synchronized via serum withdrawal and the drive to proliferate is much lower, therefore the electrical stimulation response will be

larger because the majority of the cells are moving towards G1 arrest already Expectedly, there were observed differences T198-p27 protein levels between PM and DM Cells in the proliferating media showed more modest increases in T198-p27 compared to cells in differentiating media which reflect the more reserved pAMPK increases in PM cells Similarly, T198-p27 levels in DM conditions mirror the larger increases seen in pAMPK of DM cells

Although, the protein profile differences between PM and DM conditions are suggestive of an increased rate of differentiation (that is cell cycle arrest is a prerequisite for myogenesis), COX IV and MyoD protein levels were measured to see the effect if any the premature cell cycle arrest had on differentiation modified proteins As previously discussed, COX IV was used as an indirect indicator of differentiation status COX IV is a subunit of the electron transport chain located in mitochondria Previous reports indicate that as differentiation progresses, COX IV levels also increase [1] In PM cells COX IV levels reached a significant increase at day 3 of electrical stimulation and began to fall This fall can be attributed to the controls cells 'catching up' in a sense to the stimulated cells differentiation status DM cells however experienced no change in COX IV levels over days of electrical stimulation Although the DM cells appear to have no change, it is clear from the blots (Figure 18A) that there is an increase in COX IV, however because the control cells seem to increase proportionally with the stimulated cells, when the stimulated protein content is corrected for the control cells, no change is found Most likely this can be explained by the DM growth condition Due to the fact

that the control are in DM also, their differentiation status will be more advanced than the control cells in the PM condition simply due to the serum withdrawal Having these cells already at a more advanced stage of differentiation, it is not entirely unexpected that smaller changes would be found in COX IV due to electrical stimulation in these cells The cells in PM however do show that COX IV is in fact altered due to electrical stimulation and provided some insight into the differentiation status and mitochondrial alterations in these cells COX IV is necessary for mitochondrial biogenesis and it is well documented that mitochondrial number increases with differentiation status [1] Although COX IV levels have been shown to increase with differentiation status, it is more of an indirect indicator than having a direct role in the myogenic process To gain a clearer understanding of the differentiation status of these cells MyoD levels were also measured As previously mentioned MyoD is a MRF family member which is expressed to induce differentiation [9] Figure 18 shows that significant increase in both PM and DM cells over days of electrical stimulation Again the differences between PM and DM conditions are easily explained by the presence of growth factors in PM That is there are competing actions occurring The MyoD protein levels are more telling of the differentiation status of these cells as it is a measure of a protein which even on its own, can induce myoblast differentiation [94] Although further investigation into the myogenic status of these cells is required, these data give evidence for electrical stimulation increasing the differentiation status of myoblasts

Electrical stimulation induced myoblast cell cycle arrest is AMPK dependent

In order to investigate the true role of AMPK in the stimulated-induced changes in p27 and cyclin E, cells in PM and DM were treated with compound C to inhibit AMPK. AMPK has been implicated to be the signalling pathway responding to electrical stimulation and determination of the role of AMPK in the response to electrical stimulation was investigated. Electrical stimulation induced AMPK alterations in both PM and DM conditions, and compound C reduced or eliminated these electrical stimulation-induced changes. Previously published data found a comparable amount of inhibition due to compound C on pACC (a direct target of AMPK). Another direct downstream target of AMPK is p27. Compound C induces strong decreases in T198-p27 and total p27 protein levels following electrical stimulation, which suggests that AMPK is mediating this p27 response to electrical stimulation. The depression of p27 caused by compound C indicates that cell cycle progression is continuing normally in response to electrical stimulation, as it is likely that no other CKI (p21 or p57) would be upregulated to compensate. Although C2C12 cells treated with compound C and AICAR have previously been shown to have no effect on p21 or p53 levels[81], this needs to be evaluated directly in my electrical stimulation model. Given this published finding, it is unlikely that the cells in my experiments are undergoing apoptosis in response to electrical stimulation and compound C. In addition to these effects on p27 of compound C, cells treated with compound C also

had significantly higher cyclin E protein levels than those treated with DMSO in PM and DM cells. When AMPK is inhibited the electrical stimulation induced cell cycle arrest does not occur pointing to AMPK as a key mediator in the C2C12 response to electrical stimulation.

Calcium may be involved with electrical stimulation dependent AMPK activation in myoblasts

The final portion of my thesis was to evaluate whether calcium is involved in the response of proliferating and differentiating myoblasts to electrical stimulation. Electrical stimulation activates AMPK to phosphorylate p27 on T198 which ultimately leads to an increase in total p27 levels. This increase in p27 is met with a decrease in free and functional cyclin E resulting in cell cycle arrest. One of the major activators of AMPK in muscle cells is the change in the AMP:ATP ratio that likely results from cross-bridge cycling. However, myoblasts cannot contract so it is unlikely that electrical stimulation of myoblasts causes a large alteration in the AMP:ATP ratio. Thus, another mechanism must be responsible for the observed electrical stimulation-induced changes. Ca^{2+} has been shown to activate CaMKK, which then can phosphorylate AMPK and electrical stimulation of excitable cells has been shown to increase intracellular calcium levels[1]. As such, I investigated the role of calcium in the observed electrical stimulation-induced changes in sell cycle regulatory proteins. Any electrical stimulation-induced increases in calcium were prevented by

treatment with BAPTA-AM, a chelator of intracellular calcium, at a concentration previously shown to prevent electrical stimulation-induced effects on *mitochondrial proteins in myotubes*[1] When treated with BAPTA BAPTA-AM treatment virtually abolished all of the electrical stimulation-induced effects on p27, T198p27 and pAMPK These results implicate calcium as a major source of the AMPK activation in myoblasts in response to electrical stimulation

Calcium and CAMKK have previously been implicated in the activation of AMPK, as CaMKK was responsible for AMPK activation due to α-lipoic acid and this response was inhibited by BAPTA treatment [95] Since an increase in intracellular calcium activates CaMKK, which can then phosphorylate AMPK, chelating the intracellular calcium will inhibit any CaMKK activation

The data obtained from these experiments have provided enough information to develop a working model of the signalling cascade which is responsible for the cell cycle arrest This working model can be seen in Figure 20 Electrical stimulation causes cell cycle arrest in C2C12 myoblasts, and that this cell cycle arrest is AMPK dependent Additionally, there is evidence that the electrical stimulation resulted in an increased differentiation status Finally, calcium may be the upstream regulator of this signalling cascade Implicating calcium in this process is extremely important as this opens many more doors for further research to be completed in this project It is well known that calcium is pivotal to other cellular operations in muscle, specifically contraction Fully understanding calcium's role in cell cycle inhibition may link a relationship

between cell cycle arrest, differentiation status and muscle contraction

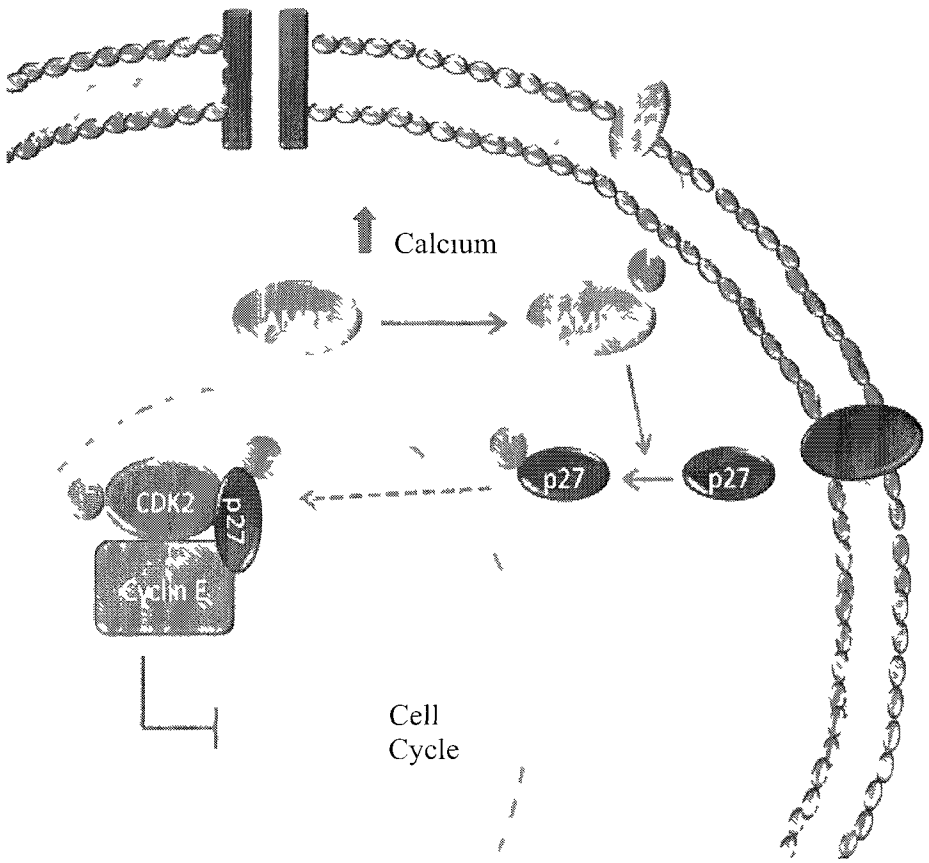

Figure 20. Current working model based upon data collected from this study Electrical stimulation may cause an increase in intracellular calcium which through CaMKK phosphorylates AMPK Active AMPK then phosphorylates p27 on T198, stabilizing it and subsequently allowing it to enter the nucleus of the cell to bind to the active cyclin E/CDK2 complex, which decreases the amount of free cyclin E, causing cell cycle arrest

PRACTICAL APPLICATIONS OF RESULTS

The work completed in this thesis has medically functional results which could lead to a better understanding and better treatment of muscle injury as well as muscle related diseases. If muscle tissue is injured resulting in satellite cell activation and the system is subsequently stimulated via contraction (exercise), premature cell cycle cessation in the satellite cells may occur and lead to premature and dysfunctional myotube formation and possible fusion. My results show that electrical stimulation *in vitro* results in cell cycle cessation and premature myotube formation resulting in an increased expression of myogenic necessary proteins. This obviously is not ideal as this could lead to an inability to heal the muscle injury which could result in chronic muscle problems.

Similar work has been completed *in vivo*; that is electrodes were placed on the peroneal nerve to induce muscle contraction in the skeletal muscle. Satellite cell activation as well as myogenic proteins were measured and it was found that the satellite cells were indeed activated via *in vivo* electrical stimulation of the peroneal nerve [96]. This paper gives *in vivo* relevance of my thesis as it is possible to activate satellite cells via electrical stimulation. My thesis then provides the evidence that even though myoblasts are non contracting cells, they are excitable and electrical stimulation/activation can cause intracellular signalling cascades to occur which lead to cell cycle arrest and subsequent premature myotube formation.

FUTURE DIRECTIONS & LIMITATIONS

This study is the first step in understanding the molecular signalling relationships in which activation of myoblasts may lead to cell cycle cessation and accelerated differentiation. As is the case, there are ways in which this study can be made stronger. To further strengthen the evidence that electrical stimulation has caused cell cycle arrest, FACS analyses can be completed to confirm G1 arrest is in fact taking place. Although previous reports have seen that an increase in p27 and a decrease in cyclin E is associated with G1 cell cycle arrest [90], confirmation of these results would only strengthen this report. Additionally, an immunoprecipitation assay could be completed for T198-p27 to determine if the p27 which is binding to cyclin E has been phosphorylated by AMPK. This would identify whether T198-p27 has a greater affinity of binding to cyclin E over unphosphorylated p27. This would implicate T198-p27 as a stronger inhibitor of cyclin E than p27.

Further insight into the differentiation status of these cells would also prove to be useful information. Initially I had planned to also look at myogenin levels within these cells for a more complete story, however due to antibody issues, this remains unchecked. The myogenin levels will provide a greater understanding of the differentiation status of these cells. Measuring levels of myosin heavy chain (MHC) would also provide a greater understanding of where the cells are along in the myogenic process. Furthermore, it would be interesting to compare typical myotubes and the premature myotubes formed due to electrical stimulation.

Determining if the electrical stimulation induced myotubes are functional would be an interesting aside to this study

Most obvious, the BAPTA experiments should be completed in the PM cells as well as in DM cells Although these data provide exciting trends, the experiments should be completed in full This information would solidify the signalling cascade proposed in Figure 20 In the more extended future, this model could be examined in animals using an exercise model or a electrical stimulation model An *in vivo* investigation would allow to see if the same results would occur in a whole body environment with many other factors influencing the system

There are limitations to any study and it is important to identify them to strengthen future experiments The system used for all experiments was an *in vitro* setup One of the most positive qualities of the *in vitro* system is the control the experimenter has on the system as a whole There are no external environmental conditions which could be affecting the results In this sense the probability that any changes seen within the *in vitro* system are due to anything but the treatment is very slim I know that the changes in protein expression of various proteins is due my treatment and nothing else The downside of this system, intuitively, is that *in vitro* conditions are not an ideal representation of how cells may respond in a whole body situation There are many processes which may occur *in vivo* that may alter the results drastically However, this information is important to learn to be able to generate any applicability within the experiments However, this study being the first of its kind, an *in vitro* system

is more ideal to begin to understand the signalling cascades which are occurring.

Overall, this thesis gives insight into the mechanisms underlying muscle differentiation in response to injury. Further investigation would possibly identify other key players in this cascade and further show the importance of this work in the muscle injury/regeneration field.

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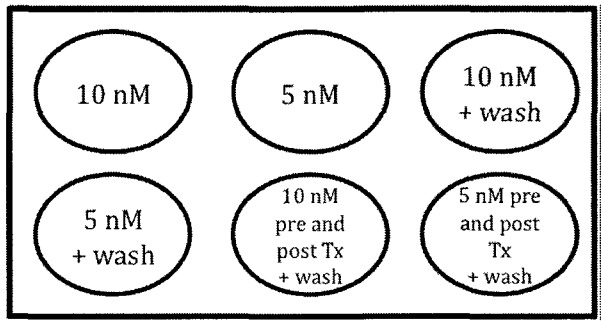
A



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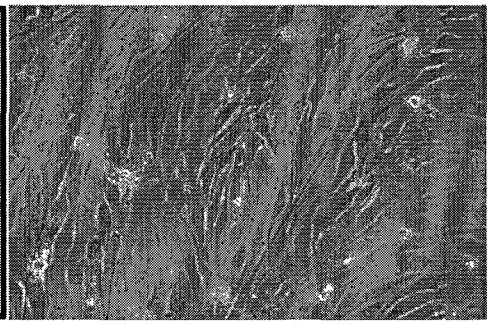

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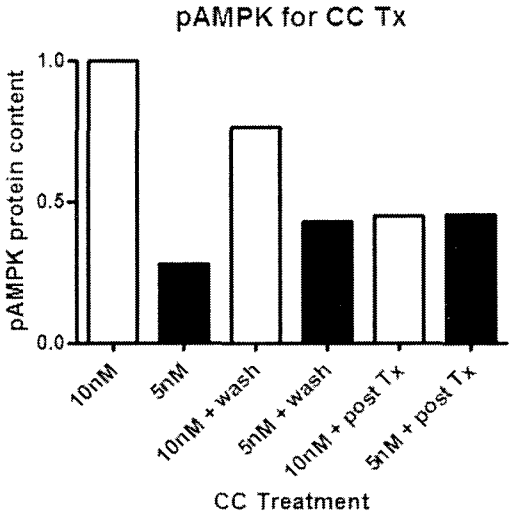

Appendix 1. Compound C concentration optimization experiment. A: optimization experiment setup. B: microscopic picture of C2C12 myoblasts after 3 days of compound C treatment at 5 nM. C: Protien content of various experimental samples measured for pAMPK after 3 days of compound C treatment.