The Immediate Early Response of Proliferating Myoblasts to One Bout of Electrical Stimulation

Matthew Triolo

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Abstract

The maintenance of muscle mass is important across the lifespan. The activation of satellite cells, followed by their proliferation and subsequent differentiation is important in this maintenance. Cell cycle arrest must precede differentiation, and preservation of the molecular networks involved within the regenerative process are necessary. Electrical stimulation is a common method of altering activity within a cell, and is known to alter the phenotype of myogenic cells. This thesis looks at the immediate effects of electrical stimulation on proliferating C2C12 myoblasts, in order to determine what induces the long term reductions in cell number associated with electrical stimulation. The results indicate that stimulation alters intracellular processes within these cells, promoting cell cycle arrest and autophagy-mediated cellular remodelling, explaining the long term reduction in cell number associated with stimulation. The research conducted is important in our understanding of muscle regeneration and muscle health.

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List of Abbreviations:

ADP	Adenosine diphosphate
AICAR	5-Aminoimidazole-4caroxamide ribonucleotide
AMP	Adenosine monophosphate
AMPK	AMP activated protein kinase
ATG	Autophagy related gene
ATP	Adenosine triphosphate
bHLH	basic helix-loop-helix
CAMK	Calcium/calmodulin-dependent protein kinase
CDK	Cyclin dependant kinase
CKI	Cyclin dependent kinase inhibitor
C	Control
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ERK	Extracellular signal regulated kinase
ES	Electrical stimulation
FACS	Fluorescence activated cell sorting
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FOXO	Forkhead box O
G_0	Gap 0 phase
G_1	Gap 1 phase
G_2	Gap 2 phase
GSK	Glycogen synthase kinase

HDAC	Histone deacetylase
HGF	Hepatocyte growth factor
IGF	Insulin-like growth factor
IL-6	Interleukin-6
INK	Inhibitor of CDK4
KIP	Kinase inhibitory protein
LC3	Microtubules-associated proteins 1A/B light chain 3
LIF	Leukemia inhibitory factor
LKB1	Liver kinase B1 or Serine/Threonine Kinase 11
M	Mitosis
MAPK	Mitogen activated protein kinase
MEF	Myocyte enhancer factor
MEK	Mitogen/extracellular signal regulated kinase
MKK	Mitogen activated protein kinase kinase
MRF	Myogenic regulatory factor
MRF-4	Myogenic regulatory factor 4
mTOR	Mammalian target of rapamycin
Myf	Myogenic factor
NAD^+	Nicotinamide adenine dinucleotide
NFAT	Nuclear factor of activated T-cells
PAX	Paired box
PE	phosphatidylethanolamine
PI3k	Phosphatidylinositol-4,5-biphosphate 3-kinase
PVDF	Polyvinylidene difluoride
p38IP	p38-intereacting protein

Rb	Retinoblastoma
RMS	Rhabdomyosarcoma
RNA	Ribonucleic acid
RTK	Receptor tyrosine kinase
S	DNA Synthesis phase
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SNAP	Synaptosomal-associated protein
SNARE	Soluble NSF attachment protein receptor
Stim	Stimulated
TGFβ	Transforming growth factor beta

1.0 - Review of Literature:

Skeletal muscle makes up approximately 40% of human body mass, controls motor function and is responsible for the majority of whole body metabolism. The ability of muscle to regenerate and maintain itself is important in ensuring healthy muscle growth and functioning across the lifespan. There are many situations that arise where individuals cannot properly regenerate or maintain a significant amount of muscle mass due to injury or disease. Therefore, an understanding of how muscle properly develops and renews itself following muscle injury is important. Additionally, many efforts are being made to understand the myogenic process in order to develop a therapy to assist muscle regeneration or to create whole muscle for implantation following severe trauma or muscle wasting. Our lab has focused on electrical stimulation (ES) as a method of altering the activity within proliferating myoblasts to determine how ES affects both proliferation and differentiation. My thesis will focus on the adaptation and early responses that are important in myogenesis and cell cycle control.

1.1 - Myogenesis:

Myogenesis is a complex process in which skeletal muscle progenitor cells mature into a fully functional muscle fibre. The formation of a functional muscle fibre begins with the signalling of mononucleated myoblasts to fuse into a multinucleated myotubes. Subsequently these myotubes transform into a fully functional muscle fibre. Regulatory checkpoints are necessary for the complete transition from myoblast to myofibre, and the abundance of these checkpoints gives this process a high degree of complexity. Many signals have the ability to either promote or inhibit both proliferation

and subsequent differentiation and fusion of myoblasts into myotubes. Many of these cellular signals are not completely established or understood.

Myogenesis occurs in two broad categories. First, embryonic myogenesis is the development of muscle tissue in a growing fetus. Second, satellite cell mediated myogenesis is the regeneration of muscle in response to muscle damage. There are many similarities between the two processes, such as common transcription factors and the temporal activation of genes (219), that are key to my thesis research.

1.1.1 - Myogenic Regulatory Factors (MRFs):

At the molecular level, muscle development is regulated by myogenic regulatory factors (MRFs). The MRFs are a family of basic helix loop helix (bHLH) transcription factors, which include MyoD, Myf5, Myogenin and MRF-4 (199). The bHLH domain is required for dimerization with E-protein transcription factors. Dimerization promotes the binding of this dimer to an E-box promoter sequence (CANNTG) on the DNA (138). MRFs are thought to play an important role in muscle specific gene expression, and the E-box promoter is highly conserved within the promoter region of many muscle-specific genes (199). The MRFs are expressed in a coordinated manner, and the result is a temporal induction of muscle-specific gene products which orchestrate myogenesis (14) (Figure 1).

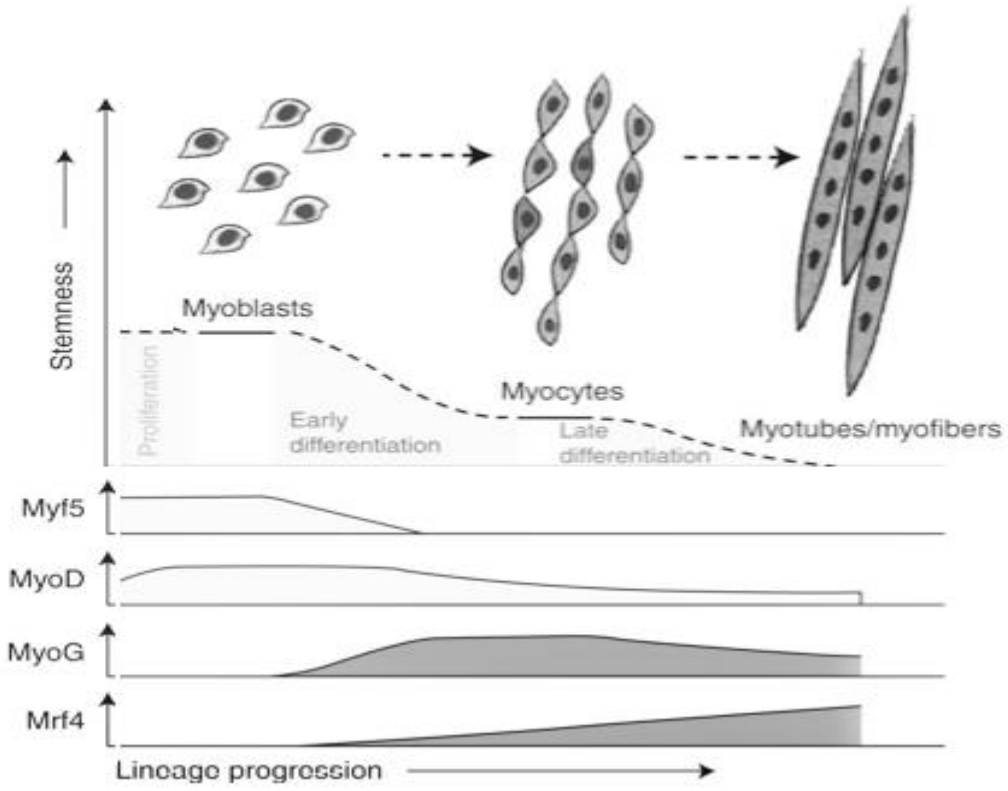

Figure 1: Myogenic Regulatory Factors (MRFs) have overlapping and unique temporal expression which regulates myogenesis. MyoD and Myf5 have overlapping expression patterns throughout the early stages of myogenesis, where myoblasts are undergoing proliferation. Myf5 is down regulated at early differentiation, whereas MyoD remains expressed throughout differentiation. Myogenin and Mrf4 have overlapping expression. Myogenin is up regulated early in differentiation, whereas Mrf4's expression slowly increases until late differentiation. As the lineage progresses from myoblast to myotubes, the stemness of these cells decreases, becoming more like a phenotypic skeletal muscle. Adapted from: C.F Bentzinger et al. 2012. *Cold Spring Harb Perspect Biol.2012;4:a008342*

Research shows that MRFs have both overlapping and unique functions in regulating myogenesis. Early research showed that MyoD and Myf-5 have a similar function, as markers of muscle lineage specification and are expressed within proliferating myoblasts. Together they are termed primary MRFs. Early work done in mice embryos showed that mice deficient in both MyoD and Myf-5 die soon after birth due to a lack of myoblasts and mature muscle (197). Interestingly, in mice that were only MyoD deficient, Myf-5 expression was increased and a normal muscle phenotype is saved (196). In contrast, Myf-5 deficient mice exhibit an abnormal muscle phenotype, which was not saved by a compensatory increase in MyoD (21). Taken together, this alludes to the overlapping and similar function of MyoD and Myf-5, but it also shows that each of the primary MRFs maintain a distinct role in myogenesis, necessary for a normal muscle phenotype to be achieved. Myogenin and MRF-4, the secondary MRFs, are also thought to be expressed in an overlapping fashion, sharing functional redundancy in promoting muscle differentiation (181). Studies conducted in mice embryos lacking myogenin demonstrated that myogenin is a lethal gene essential for muscle differentiation (151). This was not due to a deficiency in the number of myoblasts within these mice, but rather due to a lack of myofibres (73), suggesting that myogenin plays a role in promoting myoblast fusion. Comparable to the upregulation of Myf-5 in MyoD deficient mice, mice that are deficient in MRF-4 upregulate myogenin and a normal muscle phenotype is saved, alluding to the overlapping role of myogenin and MRF-4 (261). Interestingly, although previously thought to play a role in late differentiation, MRF-4 was shown to determine skeletal muscle identity in embryonic multi-potent stem cells when both MyoD and Myf5 are knocked down (94). Thus,

although functional redundancy is evident, research also shows that the MRFs hold unique functions, which are important to the myogenic process.

1.1.2 - Myocyte Enhancer Factors 2 (MEF2):

A second group of muscle transcription factors that play a pivotal role in myogenesis are myocyte enhancer binding factor 2 (MEF2) proteins. Within the family there are 4 known proteins, MEF2A-D, with A and C playing a role primarily in skeletal muscle development activating genetic programs associated will cell differentiation, proliferation, morphogenesis and survival (5, 179). Overlapping expression of the MEF2 genes occur within skeletal muscle and this orchestrated pattern of activation and deactivation which skeletal muscle development (179). The MEF2 proteins are members of the MADS family of transcription factors, which contain a highly conserved MADS-box at the N-terminus, which mediates dimerization and DNA binding, thereby promoting gene expression (18, 244). Additionally, these proteins contain C-terminal regions that act as transcriptional activation domains, which are variable among MEF2 family members (8, 17). MEF2 proteins bind to a consensus sequence within the DNA, (YTA(A/T)4TAR), where “Y” is a pyrimidine and “R” is a purine, and (A/T) is an AT rich domain within the DNA, found in the promoters of muscle specific genes, like the E-Box promoter for MRFs (63, 254). MEF2 binding to DNA is evident following mitogen depletion induction of differentiation of myoblasts but not in proliferating myoblasts (63).

Unlike the MRFs which can induce myogenesis alone, the MEF2 proteins cannot. MEF2 knockdown studies show the importance of MEF2 both embryonically and postnatally within skeletal muscle. It seems that functional differences exist between

members of the MEF2 family, and the isoform specific functions differ *in embryo* and post-embryonically. Mef2A and Mef2D knockdown in mice has little effect on skeletal muscle development. However, when Mef2C was knocked down the mice develop normally in embryo, but the postnatal muscle of these mice lack sarcomere organization due to reduction in myomesmin, which MEF2C controls (178). Additionally, both MEF2A and MEF2C transcripts are highly enriched within skeletal muscle and upregulated during myoblast differentiation and in response to muscle damage (126, 136). However, MEF2A seems to be important in regulating differentiation during muscle regeneration, whereby knockdown of Mef2A prevents proper differentiation, yet overexpression of Mef2 proteins does not induce premature skeletal muscle differentiation (47, 180). This further develops the idea that MEF2 cannot drive muscle differentiation alone, but can augment the process and ensure proper muscle development.

1.1.3 - Interplay of MRFs and MEF2s on Myogenesis:

The MRFs and MEFs exhibit overlapping expression in both embryogenesis and in adulthood (43, 153). A synergistic and cooperative action between the MEF2s and MRFs has been shown to exist (246). Through transcriptional cooperation, the MEF2 proteins potentiate the function of the MRFs (148). Mef2 is a gene target of bHLHs both *in-vitro and in-vivo,* identifying MEF2 as being under the control of the MRFs and potentiating the MRFs action in regulating differentiation (173, 237). Furthermore, the Mef2 promoter is a target of MEF2 protein, creating a positive feedback loop. Myogenin and Mrf4 gene promoters have been shown to contain MEF2 binding sites as well (31, 153) thereby allowing for MEF2 to positively feedback and amplify their expression following MEF2 expression (45). This allows for added regulation within the

differentiation program. The interplay between the MRFs and MEF2s in inducing differentiation has been reviewed briefly by Potthoff and Olson in 2007 and is shown in Figure 2.

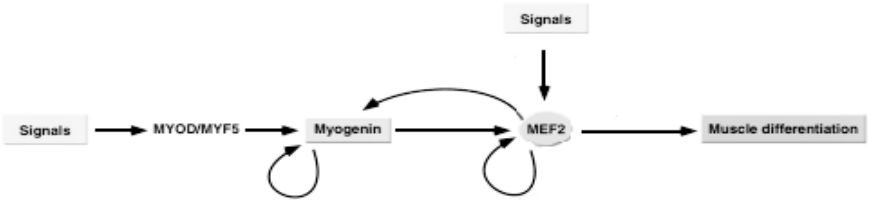

Figure 2: The MRFs and MEF2 transcription factors work together to induce muscle differentiation in developing skeletal muscle. Signals induce expression and activate the primary MRFs, MyoD and Myf5, which then act to promote the expression of myogenin. Myogenin acts in a positive feedback manner to promote its own expression, as well as promoting the expression of Mef2 which is activated by various signals within the cell. Mef2 can feedback to promote its own expression and myogenin expression, further activating the differentiation plan. Adapted from: M.J. Potthoff and E.N. Olson. 2007. *Development*.

1.1.4 - Muscle Regeneration and Satellite Cell Myogenesis:

Post embryonically, skeletal muscle is relatively stable state and in a “post-mitotic” state. Myofibres may grow throughout maturation, but the absolute number of muscle fibres remains relatively constant, although day to day muscle turnover and repair does occur. The pathways that regulate embryonic myogenesis can provide important insight into the processes that control muscle regeneration, and may be pivotal in treating myopathies where muscle regeneration is hindered. Muscle does have localized stem cells which confers a vast ability to regenerate following injury. These cells are termed satellite cells, and can divide and give rise to cells that will

ultimately be incorporated into the existing mature muscle. Satellite cells can self-renew, shown *in-vivo*, whereby a single muscle fibre was transplanted with its associated satellite cells, contributing to regeneration and replenishment (34). Furthermore, gene knockdown studies determined mechanisms involved in their asymmetric division(108).

Anatomically, satellite cells were initially found within frog muscle, and lie between the sarcolemma and the basal lamina of a muscle fibre, a phenomenon that applies to mammalian muscle as well (139). Satellite cells are easily identifiable due to their position within the muscle. They are described as “wedged” between the plasma membrane of and the basement membrane of a muscle fibre, with the satellite cell pushing on the myofibrils within the muscle cell (74, 139). The location of satellite cells is of upmost importance, whereby they have a higher density around capillaries, myonuclei and motoneuron junctions, suggesting a role that these structures play in regulation of satellite cells (74). Morphologically, satellite cells are mononucleated with highly condensed chromatin, a large cytoplasm, and very few organelles, analogous to multi-potent stem cells. Figure 3 outlines the anatomy and morphology of a satellite cell within a muscle fibre, and shows a myonuclei to draw comparison to.

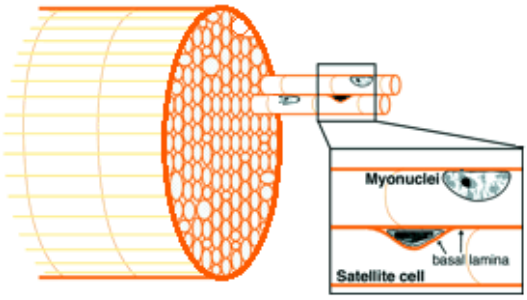

Figure 3: Quiescent satellite cells are found beneath the sarcolemma but above the basal lamina of a muscle fibre. Unlike a myonuclei, these cells are not within the muscle fibre, and these cells have highly condensed chromatin within them in comparison to myonuclei. Adapted from: Hawke and Garry, 2001. *Journal of Applied Physiology.*

In early development, satellite cells divide to provide myonuclei to growing muscle fibres, whereas in mature skeletal muscle these cells are found to be quiescent and only function when signalled to become active, as in muscle regeneration (3, 150, 205). Many models have been utilized to induce muscle injury – such as crushing the muscle, freezing the muscle, or using chemicals such as cardiotoxin to cause muscle injury (74). Furthermore, exercise has been utilized as a method of injuring muscle, showing the importance of this cell population in both muscle regeneration and the maintenance of muscle mass as an adaptation to exercise (39, 85, 194, 195, 234).

The process is strikingly similar to that of embryonic myogenesis in terms of networks that promote proliferation and subsequent differentiation and fusion of these cells. Both processes require MRF and MEF2 transcription factors to promote muscle specific gene expression. Interestingly, quiescent satellite cells from isolated muscle fibres do not express MRFs of MEF2s but do during proliferation and differentiation

(249). Quiescent satellite cells do however express the paired box transcription factor Pax7. This is different from embryonic precursors that express Pax3, which controls commitment of somatic precursors to the muscle lineage, MyoD and Myf5 expression, and migration of muscle precursor cells (11, 19, 20, 54, 135, 220). Pax7 deficient mice lack the ability to form muscle post-natally, and mutated Pax7 in skeletal muscle leads to a complete absence of satellite cells, yet when Pax7(+) satellite cells were transplanted back into the Pax7 mutant animal, muscle regeneration was rescued (200, 206). A different study found that $Pax7^{(-/-)}$ animals display a significant reduction in satellite cell content, yet sufficient satellite cells numbers exist to aid in regeneration, albeit with less efficiency (164). Together, these findings show the complexity and integral role Pax7 plays as a specification factor for satellite cells.

Activation, proliferation and differentiation of satellite cells are all integral to the regeneration and inhibition of any of these processes hinder the regeneration (183). The activation of satellite cells is promoted following muscle injury and the associated inflammatory response, where they are now similar to myoblasts. Satellite cells have the ability to respond to a variety of factors such as hepatocyte growth factor and fibroblast growth factor which promote proliferation, transforming growth factor beta which inhibits proliferation and differentiation, insulin-like growth factor which promote proliferation and differentiation, interlueken-6 and leukemia inhibition factor which promote regeneration (30, 36, 110–112, 125, 185, 208, 210, 211), reviewed in (74).

These activated satellite cells will proceed to proliferate, differentiate and fuse into the damage muscle fibre. At quiescence Pax7 is expressed, whereas MyoD and Myf5 are not (37, 206). Following activation there is an increase in the transcripts of

primary MRFs, MyoD and Myf5, with continued expression of Pax7(37, 206, 217, 249). At the onset of differentiation there is an increase in the secondary MRFs, specifically myogenin, thereby promoting differentiation (37, 217, 249). Cell culture models have shown that Pax7 decreases the transcriptional activity and stability of MyoD, thereby repressing myogenin expression, and when myogenin is ultimately elevated, Pax7 transcription is repressed (160, 161). The ratio of Pax7 and MyoD is implicated in determining satellite cell fate. High Pax7:MyoD promotes quiescence, intermediate Pax7:MyoD allows for proliferation, and when Pax7:MyoD is low and myogenin is elevated these cells differentiate (253). Differentiation is associated with increases in myogenin, MRF-4 and Mef2 expression, all of which are promoted by the increasing MyoD (15, 173, 217, 249). The ratio of increased MyoD:Pax7 associated with proliferation and differentiation may be due to decreases in Pax7, thereby alleviating the transcriptional repression it has on MyoD. A summary of the complete process and the temporal expression of the genes involved are shown in Figure 4.

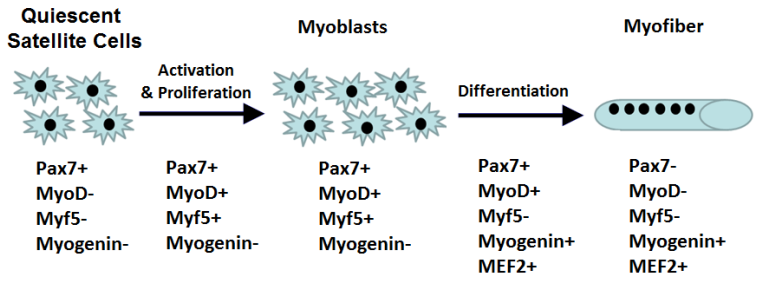

Figure 4: Gene expression of Pax7, MRFs and MEF2 differ throughout satellite cell mediated muscle regeneration. Quiescent satellite cells express only Pax7. Once activated, satellite cells proliferate the these myoblasts express both Pax7 and primary MRFs, MyoD and Myf5. After induction of differentiation, Pax7 is down regulated, whereas Myogenin and MEF2 are expressed to promote differentiation

1.2 - Cell Cycle:

During the cell cycle, a cell undergoes DNA replication followed by the division into 2 identical daughter cells (202). The cycle can be broken down into four stages. The initial growth and preparation phase is termed gap-phase 1 (G1), and in this phase the cell readies itself for DNA replication. Following this phase, the DNA synthesis phase (S) occurs, in which the DNA physically replicates, doubling chromosomal number. The second gap-phase (G2) follows and during this phase newly synthesized DNA is checked for abnormalities and the cell prepares for division. The final major stage in the cell cycle is the physical splitting of the cells which occurs in the mitotic (M) phase. Additionally, there is a G0 phase, in which a cell is no longer cycling, but has the ability to enter the cycle at G1 if prompted to (233).

1.2.1 - Function of CDKs, Cyclins and CKIs:

The cell cycle is a very tightly regulated process, and checkpoints permit or prevent the progression through the process. An overview of the process is shown in Figure 5. The eukaryotic cell cycle is regulated by the interaction of cyclin-dependant kinases (CDKs) and cyclins (134). Pairing of a CDK with its associated cyclin promotes cell cycle progression, via phosphorylation of select targets which subsequently effect downstream processes (229). Cyclins get their name because they are expressed cyclically throughout the cell cycle. Temporal waves of expression match different transition points such as G1-to-S, G2-to-M, and M-to-G0 (16). Added complexity comes from protein inhibitors of the cell cycle, termed CDK-inhibitors (CKIs). Two families of CKIs functionally inhibit the cell cycle. One is the inhibitor of CDK4 activity (INK4)-family of proteins (p15-p16, p18, p19) and the other is the kinase-inhibitory protein (KIP) -

family proteins (p21, p27, p57). INK-proteins form a complex with CDK 4 and 6, preventing their association with cyclin D(25). KIP-family proteins inactivate CDK-cyclin complexes and have higher affinity when cyclins are associated with CDKs rather than CDKs alone (67, 164, 218).

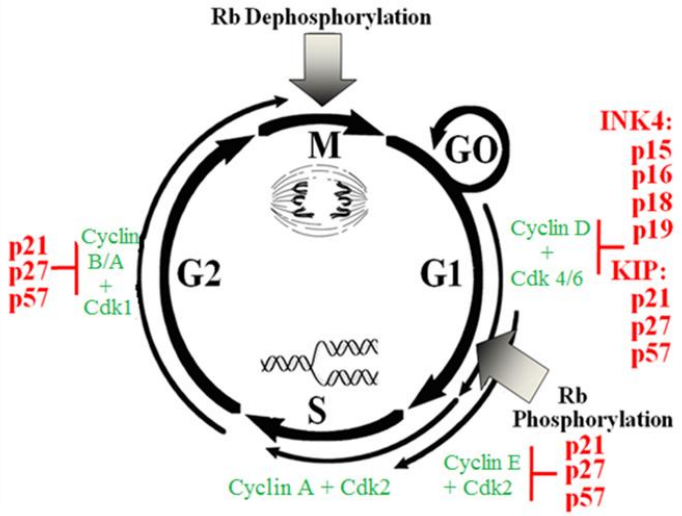

Figure 5: Stages of the mammalian cell cycle. The G0 phase is a quiescent stage, and when prompted to a cell will enter the cell cycle, beginning with G1, where the cell readies itself for DNA replication. In S-phase DNA will replicate, followed by G2, where the cell readies itself for cell division in the M-phase. Interactions of cyclins and their associated CDKs (green) promote cell cycle entry, while members of the KIP and INK family (red) act as cell cycle inhibitors, preventing progression through the cell cycle. Adapted from: Donovan and Slingerland, 2000. *Breast Cancer Res.*

1.2.2 - Regulation of the Cell Cycle:

Regulation differs at various points throughout the cell cycle, but the key to cell cycle progression is that the drive from cyclin/CDKs must be greater than the inhibition of the CKIs. During the G0 phase, all cyclin/CDK complexes are inhibited with cyclin levels being low and CKI levels being high, which prevents cell cycle entry. Additionally,

hypophosphorylated retinoblastoma protein (pRb) is inhibiting E2F transcription factors, thereby impeding the expression of genes necessary for S-phase entry (95, 130).

Cell cycle entry at the G0/G1 transition is regulated by cyclin D and CDK4/6. Prior to, and early in G1 there is an increase in cyclin D isoforms, which associate with and activate CDK4 and CDK6 (134, 182, 221). In addition, localization of cyclin D within the nucleus is important to promote cycling and S-phase entry (12). A target of the cyclin/CDK complex formed in at the G0 is pRb. Hyperphosphorylated pRb is unable to bind and inhibit E2Fs, thereby alleviating the transcriptional repression put on E2F by pRb promoting S-phase entry (9, 95). Once this point is reached, the cell will be committed to DNA duplication (202). This is the only phase within the cell cycle where both INK and KIP families of CKIs have the ability to inhibit the cell cycle(212, 213).

Cyclin E and CDK2 are important in regulating late G1, the G1/S transition, and entry into the S phase. Cyclin E expression is cyclical, reaching maximal expression in late G1 and dropping during S-phase as cyclin A levels begin to rise (104). Overexpression of cyclin E shortens G1 and promotes entry into S-phase, and silencing of cyclin E leads to abnormal G1/S transitions (157). Knockdown of cyclin E is embryonic lethal in mice, while knockdown of CDK2 is not, indicating the importance of cyclin E in cell cycle progression (60).

To progress through the G1/S checkpoint a cell must overcome the inhibition of KIP proteins in G0 and G1 (33). In addition to cyclinE/CDK2, the association of cyclin A with CDK2 is important at the G1/S transition and progression through S-phase. Inhibition of cyclin A prevents its nuclear accumulation and DNA synthesis (62). KIP

family of proteins can impede the formation of cyclinE/CDK2 and cyclinA/CDK2 complexes at this point within the cell cycle (213). p27 is elevated in quiescent cells and reduced in cycling cells, and has the ability to induce G1/S arrest through nuclear interactions with cyclinE/CDK2 or cyclinA/CDK2 (7, 156). At this stage p21 can also inhibit the cell cycle (236). Interestingly, p21 has an E-box in its promoter linking MyoD to inhibition of the cell cycle (67). In order to proceed through to S-phase, a cell must overcome this inhibition (212).

Following DNA replication, the S/G2 checkpoint is reached. Throughout G2, the cell will prepare itself for cell division, and check DNA integrity. G2 and the G2/M checkpoint are tightly regulated by the CyclinB/A/CDK1 complex. Substrates for these complexes are nuclear laminins and microtubules that aid in cytoskeleton formation to assist in the division process (98). KIP cell cycle inhibitors have the ability to inhibit the cell cycle at this point. If DNA damage is detected, tumor suppressor p53 is expressed and transcriptionally promotes p21 expression thereby arresting the cell cycle (140). Cancer cells treated with anti-cancer agents show elevations in p21 and p27 and cell cycle arrest at G2/M (247). Further, in response to DNA damage, p27 deficient mice lack cell cycle arrest at the G2/M, and accumulate damaged DNA, indicating a role of both p21 and p27 in arresting the cell cycle in response to various cues in G2 (172). Following the G2/M checkpoint, a cell will divide and the daughter cells will proceed through the process again as long as the appropriate signals are present.

1.2.3 - Myogenesis and the Cell Cycle:

The shift from proliferation to a cyclically arrested and terminally differentiated state is integral to myogenesis. First, quiescent satellite cells must be activated and enter the cell cycle. These proliferating cells are in a state of continuous cell cycling and cell cycle arrest must precede differentiation for proper progression of myogenesis. An understanding of the cell cycle and its regulation throughout myogenesis has important implications in muscle regeneration. A summary of how the cell cycle and myogenesis function together is shown in Figure 6.

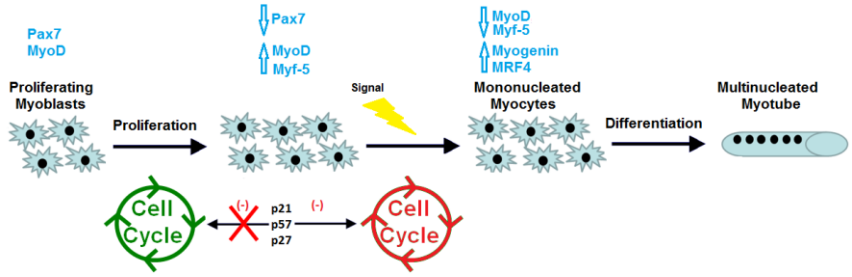

Figure 6: The interplay of the cell cycle and myogenesis. Following activation of quiescent progenitors, myoblasts will upregulate MyoD and Mf5 and proliferate. At this time the cell cycle proteins are overriding the inhibition put on them by cell cycle inhibitors (p21,p57 and p27). Following a signal, cell cycle arrest will precede differentiation. These terminally differentiated mononucleated myocytes will begin to express Myogenin and MRF4 which will promotes fusion and the formation of multinucleated myotubes

As with all cell cycle entry, CKI inhibition is overcome by cyclin/CDK drive which promotes the proliferation of satellite cells. It has been proposed that quiescence is regulated by Myf5 because its expression is high in G0 and G2, whereas cell cycle entry and early proliferation of satellite cells is regulated by Pax7 and MyoD, whereby MyoD is highly expressed in G1 (102, 227). Furthermore, MyoD is a transcriptional activator of p21, allowing it to control proliferation post-activation (67, 70, 102, 160, 227). However,

in activated and proliferating satellite cells MyoD is inhibited by Pax7 (256). Furthermore, CyclinE/CDK2 and CyclinD/CDK4 complexes, which are elevated as the cell is cycling, promote the degradation of MyoD, thus reducing p21 expression and MyoD induced cell cycle arrest and therefore the onset of differentiation (66, 227, 259). Interestingly, AKT activation during proliferation stabilizes p21 by phosphorylation on T155 and S146, allowing p21 to assist in the assembly and activation of cyclinD/CDK4/6, promoting cell cycling (121). Furthermore, cyclin D null mice lack the proliferative capacity of their wild type counterparts, making it important in satellite cell cycle entry (129). Cyclin D prevents MyoD from transcribing p21 expression, thereby preventing MyoD induced onset of differentiation (204, 206). In addition, CDK4 can block myogenin and MEF2 activation, further preventing premature differentiation (117).

When sufficient conditions are met the differentiation program will be initiated. However, this is dependent on cell cycle exit. CKI activity must be greater than that of the cyclins/CDKs and the negative regulation of MyoD and p21 that promoted proliferation and prevented premature differentiation must be alleviated (103). Importantly, $MyoD^{-/-}$ myoblasts display delayed differentiation(187), whereas $Myf5^{-/-}$ myoblasts exhibit early differentiation, indicating that MyoD is important in promoting cell cycle withdrawal and the onset of differentiation, likely through p21 (149, 198). Following the induction of differentiation, cyclins A, B, D1, and E are down regulated, promoting cell cycle withdrawal (86). The reductions in cyclin D1/2 are GSK-3β mediated and these reductions are integral in promoting differentiation because forced elevations of cyclin D1/2 induces continued proliferation of myoblasts (40, 168). In contrast cyclin D3 is unchanged at the onset of differentiation, however it promotes

skeletal muscle specific cell cycle arrest through MyoD and p21 (28, 70, 75). Although p21 is a transcriptional target of MyoD, it is expressed in myoblasts even when MyoD is knocked down, suggesting that other factors contribute to p21 expression (169). Following the onset of differentiation, p57 is also upregulated and promotes cell cycle arrest through inhibition of cyclinE/CDK2, likely by preventing the associated breakdown of MyoD by cyclinE/CDK2 (189). Interestingly, p21/p57 double knockout mice show over proliferation of muscle precursors and lack terminal differentiation leading to deficits in muscle mass (260). In addition to p21 and p57, p27 is upregulated in differentiation and is implicated in skeletal muscle cell cycle arrest (32). Its expression is transient in the myotomes of mice embryos, important in short term cell cycle exit by enhancing MyoD-initiated myogenesis, whereas in fully differentiated myocytes expression of p27 is elevated (141, 255), likely holding the cell in an arrested state. Recently, loss of p27 was shown to promote proliferation, and impair satellite cell renewal following injury and impairs differentiation, potentially through its effects on MyoD (29, 141). The CDKs and CKIs are precisely regulated and work co-operatively with myogenic factors to ensure that cell cycle arrest precedes differentiation. It is now, following withdrawal from the cell cycle, that the expression of myogenin and other differentiation related factors are elevated and differentiation occurs (6).

1.3 - Myogenic Regulation:

There are regulatory inputs that muscle progenitors receive which have the ability to promote or prevent proliferation, cell cycle arrest, and differentiation. The myogenic program has the ability to be altered by extracellular signalling which transduces its signal internally. This section will review some of the signalling networks that exist within skeletal muscle myoblasts and the potential effects of altering such networks.

1.3.1 - Regulation by AKT:

One source of regulation is through AKT. In a sub confluent and growth factor rich state, AKT is thought to promote cell cycle progression, whereas at confluence and growth factor poor environments, it is thought to promote cell cycle exit and differentiation (103). Thus keys to regulating AKT may be cell-to-cell contact and growth factor availability (103). An overview of AKTs differential effects is seen in Figure 7.

AKT promotes proliferation in a variety ways. One way it does so is through the regulation of p27 localization and stability. p27 is phosphorylated by AKT on the T157 residue, stabilized it within the cytoplasm, preventing its nuclear import and association with CDKs and cyclins (123). Furthermore, AKT promotes proliferation by preventing p27 gene expression through inhibition of FOXO transcription factors (22, 30, 105, 133). FOXO can also reduce cyclin D levels directly or through GSK-3β mediated phosphorylation of cyclin D on T286, which promotes its nuclear export and degradation (40, 214). Thus, inhibition of FOXO and GSK-3β by AKT will promote cycling (204). In addition, AKT stabilizes p21 by phosphorylation on T155 and S146, which assists in the assembly and activation of cyclinD/CDK4/6, promoting cell cycling (121). Furthermore,

AKT1, but not AKT2 is required for proliferation, by phosphorylating p21 on T145, preventing AKT2 mediated cell cycle arrest (79). In conclusion, AKT activation can act on FOXO, p27, p21, and promote proliferation.

AKT activation promotes cell cycle exit and subsequent differentiation when myoblasts reach confluence and proper growth conditions are met (103). AKT2 prevents the proliferative effects of AKT1 on p21 and promote cell cycle exit (79, 80). Furthermore, AKT acts on pathways which destabilize cyclin D and promote cell cycle exit and can promote differentiation potentially though MyoD (61, 80). Myogenin activity is pivotal for the terminal differentiation of myoblasts and is necessary for skeletal muscle fibre formation and is under the control of AKT through GSK-3β (188). Constitutively active GSK-3β represses myogenin transcriptional activity without affecting its protein level. Overexpression of GSK-3β represses myogenin, thereby preventing differentiation in C2C12 myoblasts(41). Active AKT inactivates GSK-3β through phosphorylation on the Serine 9 residue, alleviating the repression it has on myogenin expression which promotes differentiation (232). IGF-1 signaling pathways that activate AKT lead to inhibition of GSK-3β activity and promotes differentiation (38, 167, 191). Furthermore, in response to AKT activation, GSK-3β may target MEF2 post-translationally, as the inhibition of GSK-3β enhances MEF2A/D expression and transcriptional activity (42).

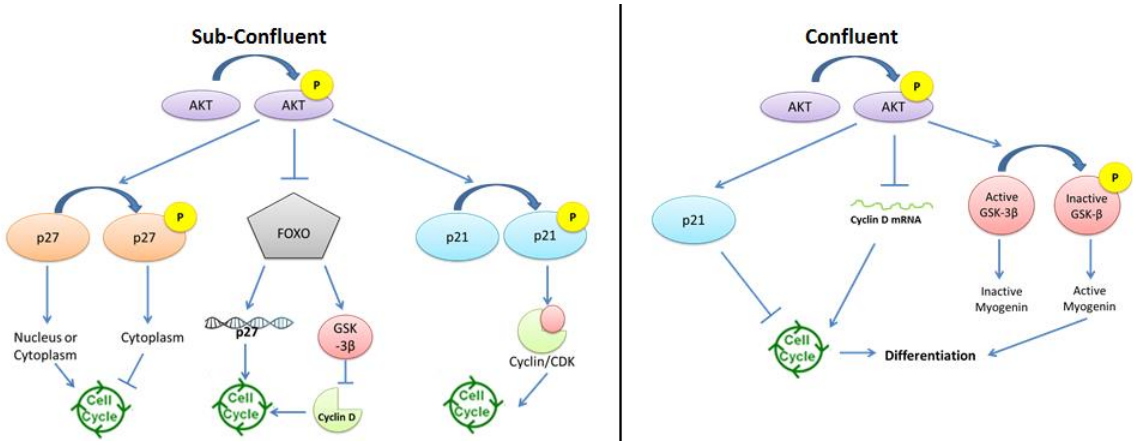

Figure 7: Differential regulation of myogenesis by AKT in a proliferative sub-confluent state (left) and a confluent state (right). In a sub-confluent state, AKT promotes cell cycling and proliferation, whereas in a confluent state it promotes cell cycle exit and differentiation.

1.3.2 - Regulation by Mitogen-Activated Protein Kinases (MAPKs):

Mitogen-activated protein kinases (MAPKs) such as extracellular signal-regulated kinase 1 and 2 (ERK1/2 or p42/p44-MAPK) and p38-MAPK also regulate the myogenic process. Both MAPK families are activated by mitogen stimulation, but their activation networks and functions differ, whereby the ERKs act on proliferation and differentiation, and p38-MAPK are implicated in differentiation only.

ERK1/2 (p42 and p44) are MAPKs that are activated in response to growth factors and mitogens. A receptor tyrosine kinase transduces an extracellular signal and through sequential phosphorylations of Raf1 and the MAPK-kinases MEK1 and MEK2, which leads to phosphorylation of the ERKs (84, 163, 190). Phosphorylation of ERK1/2 allows for its nuclear translocation where it activates transcription factors via

phosphorylation(101). The ERKs are thought to play a dual role myoblast proliferation and differentiation because of their ability to respond to stimuli for both proliferation and differentiation, and phosphorylate many targets. Early in the myogenic program ERK activity is implicated in being stimulatory to proliferation and inhibitory to differentiation, although the reverse is true late in the myogenic program, suggesting different ERK mediated mechanisms are involved in proliferation and differentiation (1, 144, 245).

The ERKs have been implicated in the promotion of proliferation in fibroblasts and adipocytes through cell cycle regulation (166, 177), and this is true within myoblasts as well. In myoblasts the forced inactivation of ERK1/2 prevents proliferation and blocks cells at G1/S and the activation of ERK1/2 promotes proliferation (78, 88). This is through increasing cyclinD1 expression (116). In addition, ERK1/2 decreases p27 protein levels through an ERK-dependant phosphorylation-mediated degradation of p27 and in MCF7 breast cancer cells ERK1/2 activation promotes p27 nuclear export and degradation, although other research indicates otherwise (53, 107, 187). This shows the complexity of the system and that further evaluation is required to determine the specific role the ERKs play temporally in myogenesis. ERK1/2 may also prevent the onset of differentiation by inhibiting MEF2 nuclear translocation and preventing MyoD and myogenin from activating differentiation specific genes via Fos and Jun activation (120, 242). Together this indicates the importance of ERK in maintaining a proliferative state within myoblasts although the mechanisms are not fully understood and require further evaluation.

ERK is also important in promoting differentiation as well, although the role it plays in the transition between proliferation and differentiation is not well understood.

However, recent evidence suggests that the shuttling of ERK between the nucleus and cytoplasm is what drives proliferation (nuclear) or differentiation (cytoplasmic) (142). Following cell-to-cell contact and mitogen depletion, ERK activation decreases, which may be mediated by AKT activation (64, 192, 235, 250). However, evidence suggest that ERK activation substantially increases to assist in differentiation of myoblasts (64, 163). Moreover, FGF inhibition prevents ERK1/2 activation and promotes cell cycle exit and differentiation (142). Likewise, inhibition of MEK and thus ERK1/2 promotes differentiation associated with an increase in MyoD and myogenin (4). The inactivation of ERK1/2 at the onset of differentiation promotes the expression of MyoD (64), through reduced AP-1 activation, which then leads to increased p21 expression (163) and cell cycle arrest. Thus a decrease in ERK activity may promote the onset of differentiation. Further, ERK2, and not ERK1, is required for terminal differentiation of myoblasts(119), developing the idea that differential regulation of the ERKs may be important in myogenesis. All together the research indicates the important role ERK activity plays in early proliferation, differentiation and the transition between the processes. The intricacies of the system are not well defined and warrant further investigation.

Another MAPK is p38-MAPK, which is activated by an upstream MAPK Kinase (MKK or MAPKK), specifically MKK3 and MKK6(46, 84, 190). Four isoforms of this protein exist; α,β, γ, and δ, with the α isoform being most important within muscle (257). The kinase activity of p38 increases throughout differentiation (162) where it is thought to promote cell cycle exit and activate proteins associated with differentiation. Knockdown of p38α in cultured and neonatal muscle induces continued proliferation and delayed maturation of musculature (174). Cell cycle exit may be promoted by p38

through inhibition of cyclinD1 transcription and protein expression, and knockdown of p38α leads to elevated cyclinD transcription through activation of JNK/cJun mediated transcription (116, 174). Most importantly, MEF2 proteins contain MAPK phosphorylation sites that are only responsive to p38-MAPK and not ERK1/2 (162). p38 activation increases the expression of MEF2 responsive genes, and MEF2A seems to be the preferred substrate for active p38, but all MEF2 proteins are able to be activated by p38 (162, 262). In concert with this, MEF2C phosphorylation by p38 of enhances its transcriptional activity (71). Inhibition of p38 in myoblasts prevents the differentiation program, while activation of p38 stimulates muscle differentiation, through its ability to indirectly activate MyoD and directly phosphorylate and activate MEF2 (T312 and 319) thereby stimulating muscle differentiation (245, 251, 262). Specifically, active p38 phosphorylates E47 allowing it to dimerize with MyoD and enhances MyoD activity, and may be further mediated by p38's activation of MEF2C, a coactivator of MyoD (127, 258). p38 activity promotes the binding of MyoD and MEF2 to late-activated promoters, and forced expression of p38 early in differentiation promotes the expression of late differentiation genes (173) . Most recently, p38 activity has been shown to be important in both early and late differentiation, where inhibition prevents both the onset of differentiation and the fusion of cells in late differentiation (59). Together this suggests an important role of p38-MAPK in regulating myogenesis, more through its differentiation effects than its cell cycle effects.

1.3.3 - Regulation of the Cell Cycle by AMPK and p27:

AMPK, an energy sensitive kinase within the cell is activated when the AMP:ATP ratio is increased, which is indicative of compromised energy status. It is phosphorylated on the T172 residue, when its own phosphorylation site is exposed, by the highly ubiquitous kinase LKB1 (209, 248). A second, LKB1 independent mechanism, that activates AMPK is in response to increases in intracellular calcium (Ca^{2+}), whereby $[Ca^{2+}]_{IC}$ increases the activity of Ca^{2+}-Calmodulin-Dependent Protein Kinases (CAMKs) and in muscle cells specifically, CAMKII (76, 184, 193). Inhibition of CAMKII prevents AMPK activation in response to Ca^{2+} treatment (81, 243). This is especially important in muscle because following excitation of a muscle fibre from a motor neuron, there are transient increases in $[Ca^{2+}]_{IC}$ (27).

Classically, AMPK has been studied in functional muscle, in the context of metabolism (184). Its activation in response to stressors such as low nutrient availability or prolonged exercise are well established (143). In addition, AMPK has profound effects on the cell cycle, whereby its activation promotes cell cycle arrest (82, 152). AMPK was very recently shown to cause cell cycle arrest in G-361 human melanoma cells deficient in LKB1 via the activation of CAMKII by Ca^{2+} (50). Thus, in muscle, AMPK activation by both LKB1 and CAMKII have important cell cycle ramifications.

AMPK mediated cell cycle arrest has been found to occur at G0/G1 in various cancer cell types by increasing p21, p53, and p27 protein levels via post-translational modifications (24, 175, 186). Interestingly, increases in non-CDK bound p27 are found following growth stimulation, but decreases in non-CDK bound p27 are found in situations of metabolic stress, pointing to increased affinity of p27 for cyclin/CDK

complexes following post-translational modifications (17).One possible mechanism behind AMPK mediated cell cycle arrest is the phosphorylation of p27 on T198 stabilizes p27 and promotes cell cycle arrest through cyclin/CDK inhibition and autophagy and not apoptosis (114, 115, 122). Additionally, AMPK mediated cell cycle arrest has been shown to include p53 phosphorylation (S15 and S18) and activation, which increases p21 transcription, thereby augmenting and inducing cell cycle arrest (89).

The effect of AMPK on muscle development and myogenesis has not been deeply investigated, but AMPK has been shown to regulate the process. AMPK promotes myogenin expression, however, inhibition of AMPK reduces myogenin expression and prevents myogenesis from occurring through HDAC5 activation (55, 56). Moreover, there is a temporal expression pattern of AMPK that changes as a myoblast differentiates which may be important for satellite cell mediated regeneration (155). AMPK activation by AICAR inhibits myogenic differentiation when cells are in a differentiation medium (145). In a proliferative state in which cells were starved of glucose, AMPK activation was proposed to block differentiation by increasing intracellular NAD^+:NADH, which is sensed by SIRT1 (57). Furthermore, adiponectin treatment of proliferating C2C12 myoblasts promotes AMPK activation, differentiation and the expression muscle specific markers but not as effectively as differentiation media and similar to the effects of AICAR, however the effect was reduced by the AMPK inhibitor compound C (49). Thus the method of activating AMPK seems irrelevant, and its activation appears promote cell cycle withdrawal differentiation.

1.4 - Autophagy:

Autophagy is an intracellular degradation system, in which cellular components, such as organelles and proteins, are used for liberation of ATP. Contents are targeted, isolated in a double membrane (autophagosome) and delivered to the lysosome to be broken down and recycled for energy liberation. The process is catabolic in nature and highly regulated. Basal autophagy is conducted within cells and is important in degradation of damaged proteins and organelles to ensure proper cell function and homeostasis (96). Interestingly, there is interplay between the ubiquitin proteasome system (UPS) and autophagy, in which inhibition of one system promotes the other system to ensure targeted protein breakdown within the cell, which was found to the case in cardiac muscle (263). The UPS is thought to degrade short lived proteins, whereas autophagy is thought to degrade long-lived proteins, but many proteins can be degraded by both systems (154).

1.4.1 - Overview of Autophagy:

There are 3 types of autophagy: macroautophagy, microautophagy and chaperone-mediated autophagy (109). Macroautophagy is of interest in my thesis. Autophagy begins with the selection of cargo, followed by initiation of autophagy where a phagophore undergoes nucleation and expansion into an autophagosome, and completion of the double membrane autophagosome (13). The cellular constituents are engulphed in the autophagosome, which fuses with a lysosome, now termed an autolysosome (13, 109). The contents are subsequently broken down by the acidic contents and hydrolases of the lysosome, and energy is liberated and the by-products are exported by permeases to be reused within the cell (13, 77, 96, 109).

The process is tightly regulated by autophagy related genes (ATGs) and other proteins. The initiation of autophagosome formation is regulated by the ULK1/2-ATG13-RB1CC1 or ULK1/2-ATG13-FIP200 complex (13, 170). This complex and thus the initiation of autophagosome formation is regulated by mammalian target of rapamycin (mTOR). When mTOR is bound to this complex, it inhibits the initiation of autophagy by phosphorylating and preventing dephosphorylation of ULK1/2, which prevents FIP200 and Atg13 phosphorylation (90, 170) When mTOR dissociates from this complex, ULK1/2, are dephosphorylated and can phosphorylate FIP200 and ATG13, thereby activating autophagy (90, 93, 170). Knockdown of ATG13 and inhibition of mTOR prevent autophagosome formation making these important targets in the regulation of autophagy (90).

Nucleation of the phagophore involves the recruitment the ATG14 complex, which contains class III phosphatidylinositol 3-kinases (PI-3-Ks). The complex is made up of ATG14-BECN1-PIK3C3-PIK3R4 (170), and is negatively regulated by BCL2 binding to Beclin-1 thereby preventing Beclin-1 association with the complex (171). Knockdown or dissociation of BCL2 from Beclin-1 promotes autophagy (13, 124, 171).

Subsequently, the phagophore elongates and expands to become an autophagosome. Two conjugation systems regulate this process, the ATG12-ATG5-ATG16L1 conjugation complex and the LC3 conjugation system (170). The ATG12-ATG5-ATG16L1 is irreversibly formed by E1 and E2 activating enzymes that link ATG12-ATG5. The ATG16L1 then binds to ATG5, which promotes dimerization with an identical complex(146). This complex interacts with the phagophore membrane and the second conjugation complex, dissociating when the full autophagosome is formed

(147). The LC3 conjugation system relies on ATG4, a protease that processes LC3 into LC3-I in order for it to conjugate with the membrane of the phagophore (241). LC3-I is then activated by an E1-like enzyme (ATG7) which allows it to be processed by an E2-like enzyme (ATG3) and subsequently interacts with the ATG12-ATG5-ATG16L1 complex which acts like an E3-ligase, conjugating a phosphatidylethanolamine (PE) to the LC3, now termed LC3-II (170). LC3-II is the lipidated form of LC3 and is correlated with the extent of autophagosome formation, as it associates proportionally with the membrane of autophagosome (91, 224). ATG9 is also thought to assist in elongation by recruiting more membrane for the autophagosome.

The autophagosome is now proceeding towards completion and must fuse with a lysosome, forming an autolysosome, which involves cell trafficking, tethering, and fusion of the two membranes to allow for digestion of the contents. This is regulated by microtubules, SNARE proteins and SNAP proteins (13, 170). Figure 8 represents a summary of the autophagy pathway discussed above.

1.4.2 - Regulation and Markers of Autophagy:

Important to my work is how autophagy is involved in the response to cellular stressors, such as nutrient deprivation, starvation, damage to organelles and alterations to the intracellular components of a cell. AMPK has been shown to be an activator of autophagy, as is within electrically stimulated myoblasts (44). AMPK is also activated in situations of low energy and when active for prolonged periods it promotes autophagy (68, 83). It does so through either a TSC1/2 mediated inactivation of mTOR or

interaction with ULK1/2 (99, 100, 225). Therefore, AMPK is an important energy sensing target within a cell that can regulate autophagy in situations where nutrients are low.

Interestingly AKT, ERK1/2, and p38-MAPK can all inhibit autophagy and all are important in skeletal muscle differentiation. AKT can prevent autophagy, by activating

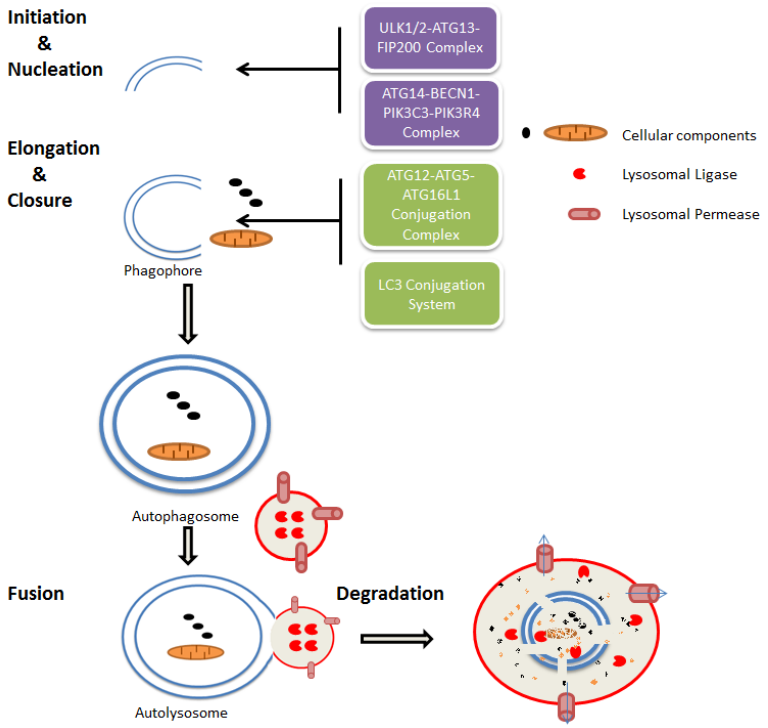

Figure 8: Autophagy is a multi-step process that begins with initiation and nucleation which are regulated by ULK1/2-ATG13-FIP200 and ATG14-BECN1-PIK3C3-PIK3R4 complexes. Elongation and closure of the phagophore follows and is mediated by the ATG12-ATG5-ATG16L1 and LC3 conjugation systems. Fusion of the autophagosome with the lysosome produces an autolysosome which degrades the contents and the by-products are released into the cytosol be lysosomal permease.

mTOR through inactivation of TSC1/2 and AMPK (69) or by phosphorylating Beclin1 which inhibits its ability to promote autophagy (239). Formation of the autophagosome is negatively regulated by p38-MAPK, as inhibition of the p38 pathway promotes autophagic cell death (97, 252). This is thought to be through p38's interaction with p38-interacting protein (p38IP) and inhibition of ATG9 trafficking (240). Interestingly, p38 phosphorylation decreases with cell starvation, and p38IP has less binding affinity to dephosphorylated p38 favoring the interaction of p38IP and ATG9 allowing for trafficking to the autophagosome and autophagy in starved conditions (240). ERK1/2 modulates mTOR signalling through TSC1/2 inactivation thereby preventing autophagy (132). However ERK also activates Beclin-1, which may be important in promoting the later processes of autophagy if they are reached (238)

Important in the study of autophagy following changes to a cellular environment are markers of autophagy. A common marker of autophagy is the conversion of LC3-I to LC3-II. This conversion is associated with autophagy, and is proportional to autophagosome formation, and is useful as a marker of autophagy, although it gives no details about flux through autophagy (91, 224). Thus in response to a treatment, measurement of LC3-I and LC3-II can give insight into the activation of autophagy.

1.4.3 - Autophagy in Skeletal Muscle and in Muscle Regeneration:

Autophagy is important in the maintenance of muscle mass and the remodelling processes following stress placed on muscle. Stress can occur in situations of nutrient deprivation, exercise and disuse (159, 231). Inhibition of autophagy promotes profound muscle atrophy with accumulation of abnormal organelles and aberrant structures, and

in response to denervation, its inhibition intensified the atrophying of muscle and the buildup of abnormal organelles within muscle (137, 158, 230). Additionally, overactive autophagy in muscle leads to the development of myopathies and muscle loss (231).

AMPK activation increases autophagy in C2C12 myoblasts and myotubes through mTOR, ULK1 and FoxO3 activation promoting autophagy related gene expression (201, 228). Endurance training stimulates autophagy in healthy skeletal muscle and without a functioning autophagy system, there is an increase in muscle wasting following submaximal exercise (65) . Following exhaustive endurance exercise there is an increase in AMPK and ULK1 activation, decease in AKT activation, and an increase in autophagy(165). Resistance exercise can also promote autophagy, through downregulation of AKT activation, upregulation of AMPK activation and FoxO3 activation(131). Together this shows that autophagy within muscle is important in cellular remodelling as part of adaptation process and to ensure proper muscle maintenance.

In terms of regeneration, declines in autophagy, such as with aging, are associated with senescence and a lack of satellite cell mediated regeneration. However, reestablishment of autophagy in these cells allows for regeneration to occur (58). One study reported that autophagy promotes satellite cell activation, and inhibition of autophagy prevents satellite cell activation (223). Autophagy is activated following the induction of differentiation in myoblasts and its inhibition prevents differentiation and fusion (51), thereby indicating autophagy as an important contributor to the correct execution of differentiation. This same study found that mTORC1 remains elevated throughout differentiation and thus autophagy in myoblasts is mTORC1 inactivation-

independent and inhibition of mTORC1 further stimulated autophagy and impaired myotube formation (51). Another study found that over active autophagy during myogenesis induces an accumulation of cell-death signalling components which may trigger apoptosis and prevent regeneration (128). Since autophagy is sensitive to metabolic changes within the cell, the increasing mitochondrial content associated with differentiation may explain the increases in autophagy during the transition between myoblast and myotube (52). Suppression of autophagy interferes with differentiation, proper mitophagy, and the ability of cells to create a mitochondrial network in myotubes (215). It is clear that mechanisms are in place to regulate autophagy and that autophagy is required for proper myogenesis, which may in part be regulated by the mitochondria.

1.5 - Electrical Stimulation:

Methods have been developed to analyze the effect of exercise on muscle cells *in-vitro* and *in-vivo*. Electrical stimulation (ES) is a commonly used model, as electrical stimulation excites muscle cells as if an action potential from a motor neuron was doing so. Initially, ES was utilized to investigate muscle metabolism and training adaptations at a cellular level in fully differentiated myotubes (35). More recently however, electrical stimulation has been studied with respect to the response of proliferating and differentiating myoblasts. Our lab has previously found that in proliferating myoblasts, long term (5day) ES elicited Ca^{2+}-dependant-AMPK-mediated cell cycle arrest accompanied by an increase in $p27^{T198}$ and cyclin E bound to p27, which together promotes cell cycle arrest (44). In addition, our lab has looked at the effects of ES on rhabdomyosarcoma (RMS) cells and found that long term stimulation induces G2 cell cycle arrest as well as activation of the autophagy regulator LC3-II (10). This was found with an associated increase in AKT activation and a decrease in pAMPK and total p27 protein levels within proliferating cells (10). Thus, it seems that ES has the ability to alter the proliferative capacity of myogenic cells, and may act as a method of inducing altered activity within a cell. This altered activity can be used to elucidate mechanisms involved in normal myoblast proliferation and differentiation as well as develop interventions for satellite cell mediated regeneration of damaged muscle.

2.0 - Study Rationale:

Previous data from our lab suggests that long term (1-5 days) ES affects proliferating C2C12 myoblasts *in-vitro*, by promoting cell cycle arrest and the induction of premature differentiation. Many important adaptations in skeletal muscle occur in the recovery period following use and stimulation, however, no experiments to date have investigated the early signalling mechanisms involved in ES-induced reductions in cell number in proliferating C2C12 myoblasts. Thus, the purpose of this study was to examine the early signalling events which lead to the observed reduction in cell number seen with long term-ES. This research aims to understand how this altered signalling in proliferating myoblasts affects the natural myogenic program and leads to the prolonged effects of ES-myoblasts previously investigated.

3.0 - Hypotheses:

The treatment of proliferating C2C12 myoblasts with one bout of electrical stimulation (ES) will:

1. Alter intracellular signalling in the post-ES recovery period that promotes cell cycle exit and cellular remodelling.
2. Alter the normal myogenic program
3. Promote autophagy and cellular remodelling of C2C12 myoblasts.

4.0 – Manuscript:

Electrical stimulation reduces cell number in proliferating C2C12 myoblasts

Matthew Triolo[1,2] and Michael K. Connor[1,2]

From the [1]School of Kinesiology & Health Science and [2]Muscle Health Research Centre

York University, Toronto, ON, M3J 1P3

To whom correspondence should be addressed: Michael K. Connor, School of Kinesiology and Health Science, York University, 347 Bethune College, 4700 Keele Street, Toronto, ON, M3J 1P3, Canada,

E-mail: mconnor@yorku.ca

4.1 – Abstract:

Long term skeletal muscle maintenance requires the activation of quiescent satellite cells in order to enter the cell cycle, proliferate to increase cell number, exit the cell cycle and subsequently fuse and mature into multinucleated muscle fibres. Regulation of this process involves the ordered expression of myogenic regulatory factors and cell cycle proteins. Appropriate signalling is important in the maintenance of healthy muscle tissue. Electrical stimulation (ES) is a method commonly employed to elicit adaptations in muscle, both *in-vitro* and *in-vivo*. Recently, ES was shown to promote cell cycle arrest in proliferating myoblasts. Our lab has focused on the long term effects of ES, but no research to date has focused on the immediate response of these cells to stimulation. Thus, we have employed a model whereby proliferating myoblasts are subjected to ES for 4hr/day followed by a recovery period. ES induced increases in AKT and p42/p44-MAPK activation, as well as an immediate increase in AMPK activation. Furthermore total p27 protein levels were reduced in response to ES, however $p27^{T198}$ was stable in the post-ES period. $p27^{T198}$ was localized predominantly within the cytoplasm and ES promoted its nuclear translocation, indicating p27s nuclear localization may regulate ES-induced cell cycle arrest. Stimulation reduced both p38 and Mef2A protein levels, with no changes in myogenin indicating that the differentiation capacity of these cells may be compromised. Interestingly, there was an immediate upregulation of autophagy marker LC3II, which was subsequently diminished by 16 hours post-ES. Together our results indicate that cell cycle withdrawal and autophagy mediated cellular remodelling occur in the response of proliferating myoblasts to ES.

4.2 - Introduction:

Skeletal muscle makes up approximately 40% of human body mass and its primary function include ambulation and metabolism. The ability to maintain and regenerate muscle mass following injury and replenishing myonuclei as they senesce are vitally important in ensuring health across the lifespan. Situations that arise in which the regenerative capacity of muscle is hindered have negative ramifications on overall maintenance and homeostasis of skeletal muscle. Since skeletal muscle is being identified as a major contributor to overall health, an understanding of the initiation and regulation of the regenerative process is important for the development of interventions to maintain the regenerative capacity of muscle and overall muscle health.

Maintenance of skeletal muscle mass is largely controlled by satellite cells (SCs; 147, 201), which lie between the sarcolemma and basal lamina of muscle fibres (74, 139). These SCs are important in both muscle regeneration and adaptation to exercise (39, 85, 194). The first step in SC response to injury is exit from quiescence and entry into the cell cycle. Once the appropriate cell numbers have been reached, the cells must exit the cell cycle prior to differentiation, a necessary step for the fusion into the damaged muscle fibre (74).

Cell cycle arrest in muscle is thought to be controlled mainly by MyoD which promotes the expression of cell cycle inhibitor p21 leading to cell cycle arrest (67, 70, 102, 216, 218, 227, 256, 259). However other cell cycle inhibitors such as p27 and p57 have been implicated in myogenic cell cycle control as well (29, 32, 141, 189, 255, 260). Electrical stimulation (ES) has been used as a method of altering the intracellular signalling within muscle both *in-vitro* and *in-vivo* (10, 35, 41, 44). Our lab has previously

demonstrated that in proliferating myoblasts long term (5 day) ES elicits Ca^{2+}-dependant-AMPK-mediated cell cycle arrest, accompanied by an increase in $p27^{T198}$ and cyclin E bound to p27. We have also shown that ES of rhabdomyosarcoma (RMS) cells induces G2 cell cycle arrest and activation of autophagy.

The induction of the basic helix loop helix (bHLH) family of myogenic regulatory factor (MRF) expression in myoblasts following SC activation promotes muscle specific gene expression regeneration (14, 15, 199, 249). Primary MRFs, MyoD and Myf5, promote muscle lineage specification and cell cycle arrest, whereas secondary MRFs, myogenin and MRF-4, promote differentiation of myoblasts into muscle(181). There is overlapping expression and temporal activity of the MRFs, which orchestrate myogenesis (14, 181). Mice deficient in both MyoD and Myf-5 display a lack of myoblasts and mature muscle (21, 196), but $MyoD^{-/-}$ mice display a compensatory increase in Myf-5, whereas MyoD does not compensate for in $Myf\text{-}5^{-/-}$ mice (21). Myogenin is necessary for muscle formation and development whereas MRF-4 may be dispensable (41, 73, 151, 261). Further, differentiation is enhanced by the MAD box transcription factors, myocyte enhancer factors 2 (MEF2) proteins (18, 244, 254). The promoter region of many muscle specific genes are responsive to both MEF2 and MRF binding (63, 136, 178, 179, 199), and a synergistic and co-operative action between the two families of proteins exists (31, 45, 148, 153). We have shown previously that ES of proliferating C2C12 myoblasts induces increases my MyoD protein but reduces myogenin protein content (44). However both are necessary for normal skeletal muscle formation and the SCs response to muscle injury (44).

Based on this evidence we subjected proliferating C2C12 myoblasts to one bout

of ES to determine what the immediate early signalling events are that induce the long term response to ES. We show that following 4 hours of ES there is a significant reduction in cell number, with immediate and maintained activation of AKT and ERK1/2, while AMPK activation diminishes during recovery. Cell cycle inhibitor p27 levels were reduced following ES, but $p27^{T198}$ was unchanged and greater within the cytoplasmic fractions of myoblasts, although a shift towards nuclear localization occurred in response to ES. Furthermore, LC3II, a marker of autophagy, was elevated immediately following ES, but returned to control levels late in the post-ES recovery period. Overall our results suggest that ES alters intracellular mechanisms involved in proliferating myoblasts, halting proliferation in what may be a p27 mediated-pathway. ES may also be promoting autophagy regulated cellular remodelling. These results may explain the effects of long-term ES on C2C12 proliferation and the potential role of ES in muscle regeneration.

4.3 - Materials and Methods:

Cell Culture

C2C12 myoblasts (ATCC, Manassas, VA) were maintained at 37^{o}C and 5% CO_2, in high glucose Dulbecco's Modified Eagle Medium (DMEM; Wisent, St. Bruno, QC), 10% Fetal Bovine Serum (FBS; Hyclone, Thermo Fisher Scientific, Whitby, ON), and 3% Anti-micotic/Anti-biotic (Wisent). Media was replenished every 48 hours in necessary experiments. Prior to plating, 6 well culture plates were coated in 0.1% gelatin and exposed to UV light for 40 minutes.

Electrical Stimulation

Cells were stimulated (10V, 5Hz, 2ms delay, alternating current direction) using a Harvard Apparatus Stimulator CS System (Harvard Apparatus Canada, Saint-Laurent, QC) for 4 hours/day for 1 day. Control cells were plated and collected at the same time as stimulated cells. Collection took place at various time points following the cessation of stimulation. The lids of the plates were fitted with two parallel platinum wire electrodes extending into the media and were connected to a cell culture stimulator. Prior to stimulation, media was added to create a total volume of 4mL to allow the electrodes to contact the media.

Cell Harvesting

Throughout the post stimulation recovery period cells were harvested. Media was removed, and cells were washed twice with cold Phosphate Buffer Saline. Cells were subsequently collected centrifuged at 2,100 xg at 4^{o}C. The pellet was resuspended in TENT buffer solution (0.2% TENT – TRIS, EDTA, NaCl, 0.2% Triton x-100)

supplemented with 1% protease inhibitor (Sigma, Oakville, ON), and 1% phosphatase inhibitor (Roche Diagnostics, Indianapolis, IN). The cells were then sonicated for 3 seconds and centrifuged at 16,100 xg at 4^{o}C. The supernatant lysate was then removed and stored at -84^{o}C for later analysis.

Cellular Fractionation

Media was removed from the cells and were washed twice in warm PBS. Cells were harvested by trypsinization, and cells were washed with cold PBS and centrifuged at 2,100 xg at 4^{o}C. The pellet was resuspended in 100 μl of a 1x TEN isotonic transport buffer. Digitonin (BioVision, Milpitas, CA; 20-30g/ml final concentration) was used to permeabilize the plasma membrane until 90-95% of cells exhibited trypan blue staining. When 90-95% of the cells exhibited trypan blue staining, the samples were centrifuged at 2,100 xg and the supernatant was collected. This supernatant represents the cytoplasmic fraction. The pellet represents the nuclear fraction and was resuspended in 100 μl of 1XTEN, sonicated, and subsequently centrifuged for 10 minutes at 16,100 xg. The cellular fractions where then stored at -84^{o}C for later analysis.

Immunoblotting

Protein concentrations within samples were determined by a Bradford Assay. 25 μg protein /lane was loaded into onto a 12 or 15% SDS-PAGE gel and run at 120V for 90-120minutes. Separated proteins were subsequently transferred onto a PVDF membrane (Bio-Rad, Mississauga, ON) overnight at 40V. Membranes were then stained in amido black and blocked in 10% milk for 2 hours. Contents of the western blot were subsequently probed for using primary antibodies overnight at 4^{o}C for: AMPK,

p-AMPK^{T172}, AKT, p-AKT^{T308}, p-AKT^{s473}, p42-MAPK, p44-MAPK, p-p42-MAPK, p-p44-MAPK, GSK-3β, pGSK-3β^{S9}, LC3, RCC1 (Cell Signaling, Danvers, MA), Mef2A, Mef2D, p38, p-p38, p27 (BD Biosciences, Mississauga, ON), t198-p27 (R&D Systems, Minneapolis, CA), Myogenin and β-Actin (Abcam, Cambridge, MA).Following incubation, membranes were washed 3 times for in Tris-Buffer Saline with Tween (TBST) and subsequently placed in anti-mouse and anti-rabbit HPR-linked secondary antibodies (Santa Cruz Biotech, Santa Cruz, CA) with 5% milk for 1 hour. Proteins were visualized using immobilon enhanced chemiluminescence substrate (Millipore, Whitby, ON) and detected or quantified on a Kodak In Vivo FX Pro Imager (Marketlink Scientific, Burlington, ON) using Carestream software. Protein loading was corrected using β-Actin.

Statistical Analyses

All statistical analyses were performed using GraphPad Prism 5 software. Unpaired student's t-tests were performed to determine if there were any statistical significance between control and stimulated conditions at a given time point. Statistical significance was found in any comparisons when differences between the means reached $p<0.05$. One-way ANOVAs were conducted to determine the effect of ES/Control, and statistical significance was found when $p<0.05$.

4.4 – Results:

Electrical stimulation reduced cell number

Immediately following ES there seems to be no difference in cell count between control and stimulated myoblasts, however by 16 hours post-stimulation there is a dramatic difference in cell number in control compared to stimulated myoblasts, whereby the control cells are reaching confluence, and the ES-treated cells are not (Figure 9).

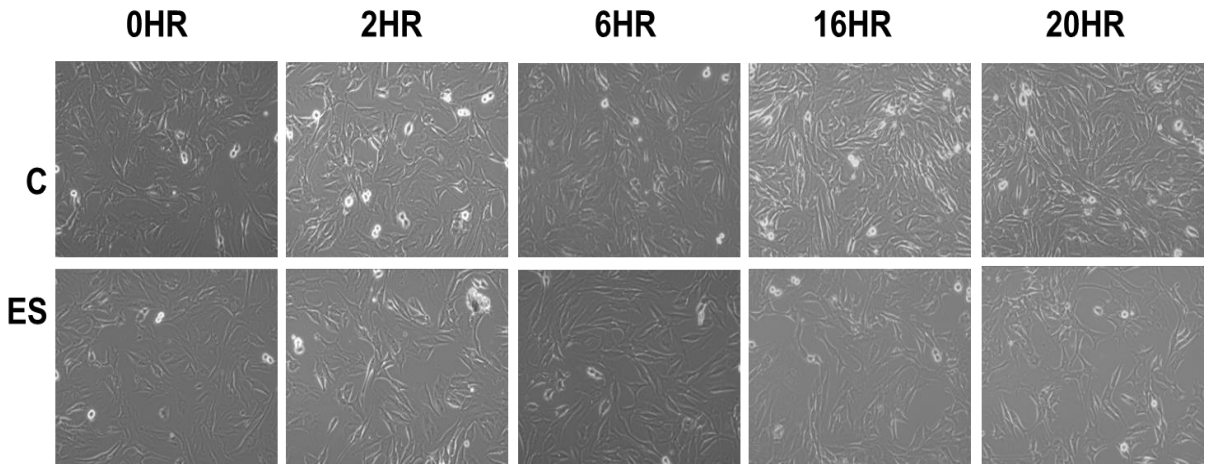

Figure 9: Electrical stimulation reduced cell number by 16 hours post-ES in comparison to time matched controls. Top are representative photos of non-stimulated myoblasts. Bottom are representative photos of ES-treated myoblasts.

Electrical stimulation activates cell signalling during recovery

Immediately following ES there was a 1.4-fold increase in pAMPK^{T172} in comparison to control, which had a peak 2.1-fold increase above control at 4 hours post-ES. pAMPK^{T172} then declined to pre-ES levels by 6 hours post-ES (Figure 10A,B).

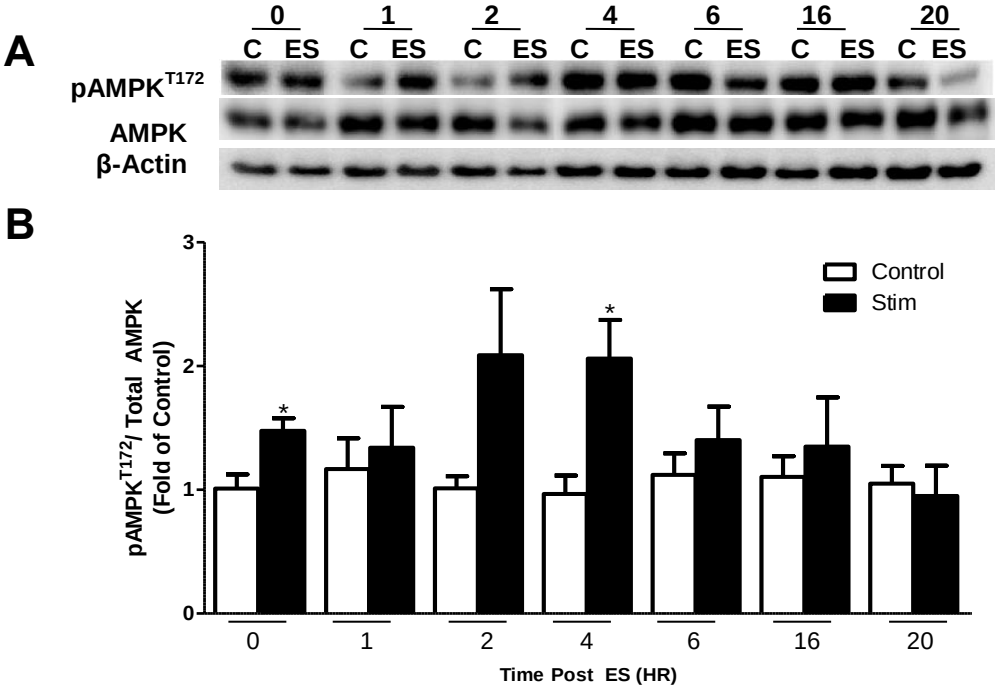

Figure 10: Electrical stimulation (ES) of proliferating C2C12 myoblasts induced early AMPK phosphorylation in comparison to non-stimulated cells. Immediately following ES AMPK phosphorylation was increased, followed by a normalization of phosphorylation to time matched control levels late in the recovery period **(B).** All values are mean ± S.E.M.; ***** $p<0.05$ compared to time matched controls (n=4-6).

Since AKT is important in skeletal muscle proliferation and differentiation, we analyzed pathway activation following ES. ES elicited an immediate 2.6-fold increase in pAKT^{T308} which reached a maximal 5.5-fold increase at 4 hours post-ES compared to non-stimulated control cells. pAKT^{T308} levels declined for the remainder of the recovery period, eventually reaching pre-ES levels after 20 hours (Figure 11A,B). Unlike pAKT^{T308}, ES caused no increase in pAKT^{S473} phosphorylation immediately after the

cessation of stimulation (Figure 11A,C). pAKT^{S473} levels rose during the recovery period and reached a significantly greater levels 2 hours after stimulation, and a peak 2.4-fold increase in comparison to non-stimulated controls 4 hours post-ES. Subsequently, pAKT^{S473} levels rapidly declined, reaching control levels throughout the remainder of the recovery period (Figure 11A,C).

AKT phosphorylates GSK-3β on S9 and this inhibitory phosphorylation alleviates GSK-3βs repression on myogenin, which in turn promotes differentiation. Despite activating AKT, ES did not lead to GSK-3β phosphorylation (Figure 12A,B). However, there is a trend towards increased GSK-3β phosphorylation between 4 and 20 hours of recovery ($p=0.10$). No changes in myogenin protein levels were evident at any time point throughout the experimental protocol (Figure 12A,C).

Mitogen activated protein kinases (MAPKs) play an important role in skeletal muscle signalling and myogenesis, thus we evaluated the effects of ES on p42 and p44 MAPK. ES induced a rapid phosphorylation of both p42 and p44, being 2.5-fold and 2.2-fold greater in stimulated cells than control, respectively (Figure 13A-C). The two MAPKs appear to respond differently throughout the post-ES recovery period. p42-MAPK phosphorylation appears to dip towards the levels seen in control cells shortly after the cessation of stimulation, but rises to levels that were 2.4-fold above control by 20 hours post-ES (Figure 13A,B). In contrast, p44-MAPK phosphorylation is elevated 2.5-fold following ES and appears to be decreasing throughout the recovery period (Figure 13A,C), although elevated above control 16 hours post-ES.

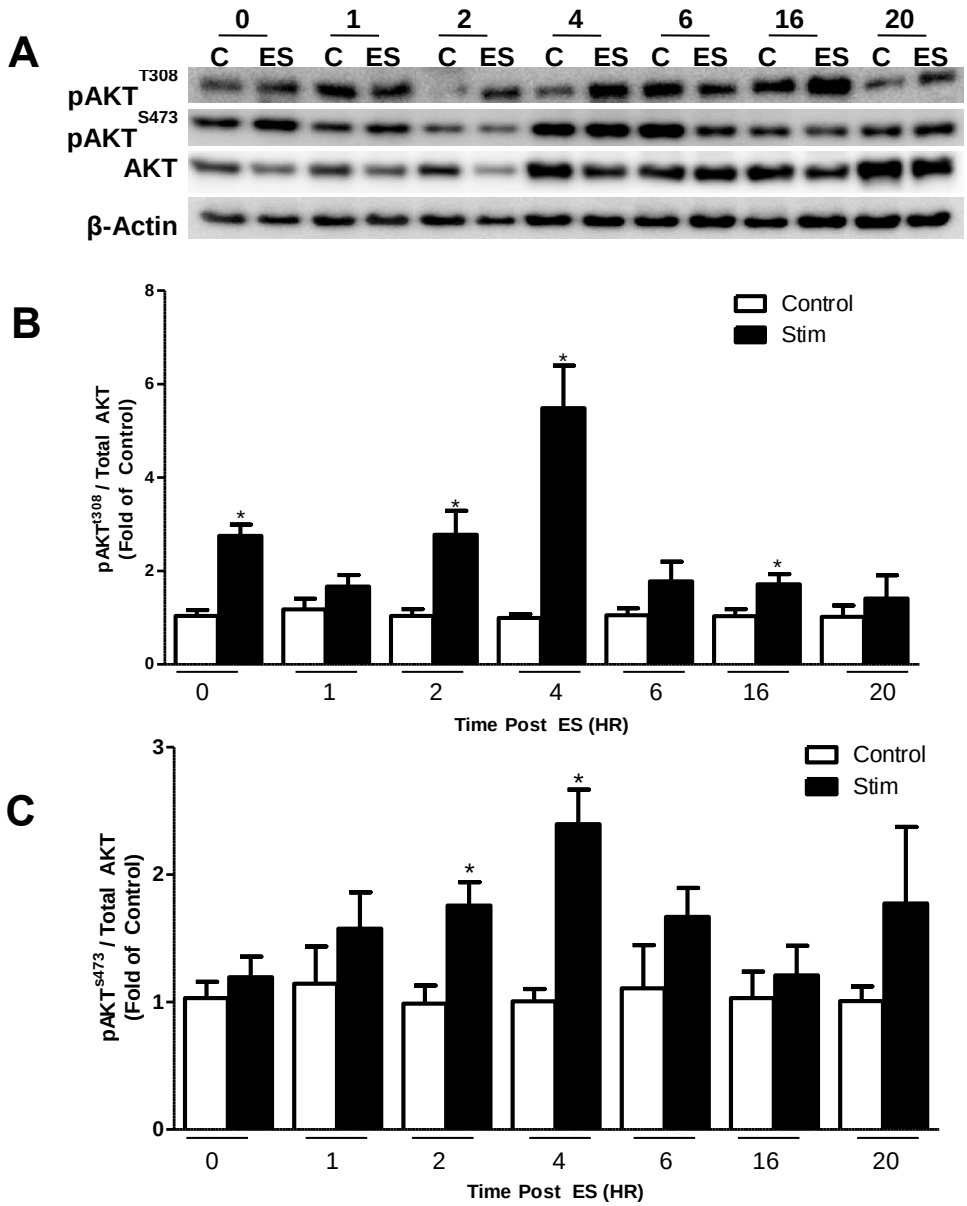

Figure 11: Electrical stimulation (ES) of proliferating C2C12 myoblasts increased $pAKT^{T308}$ and $pAKT^{s473}$. $pAKT^{T308}$ was significantly increased immediately after ES and declined following 4 hours post-ES in comparison to time matched controls **(B)**. $pAKT^{S473}$ was significantly elevated by 2 hours post-ES and remained elevated throughout the recovery period in comparison to time matched controls **(C)**. All values are mean ± S.E.M.; ***** $p<0.05$ compared to time matched controls (n=4-6).

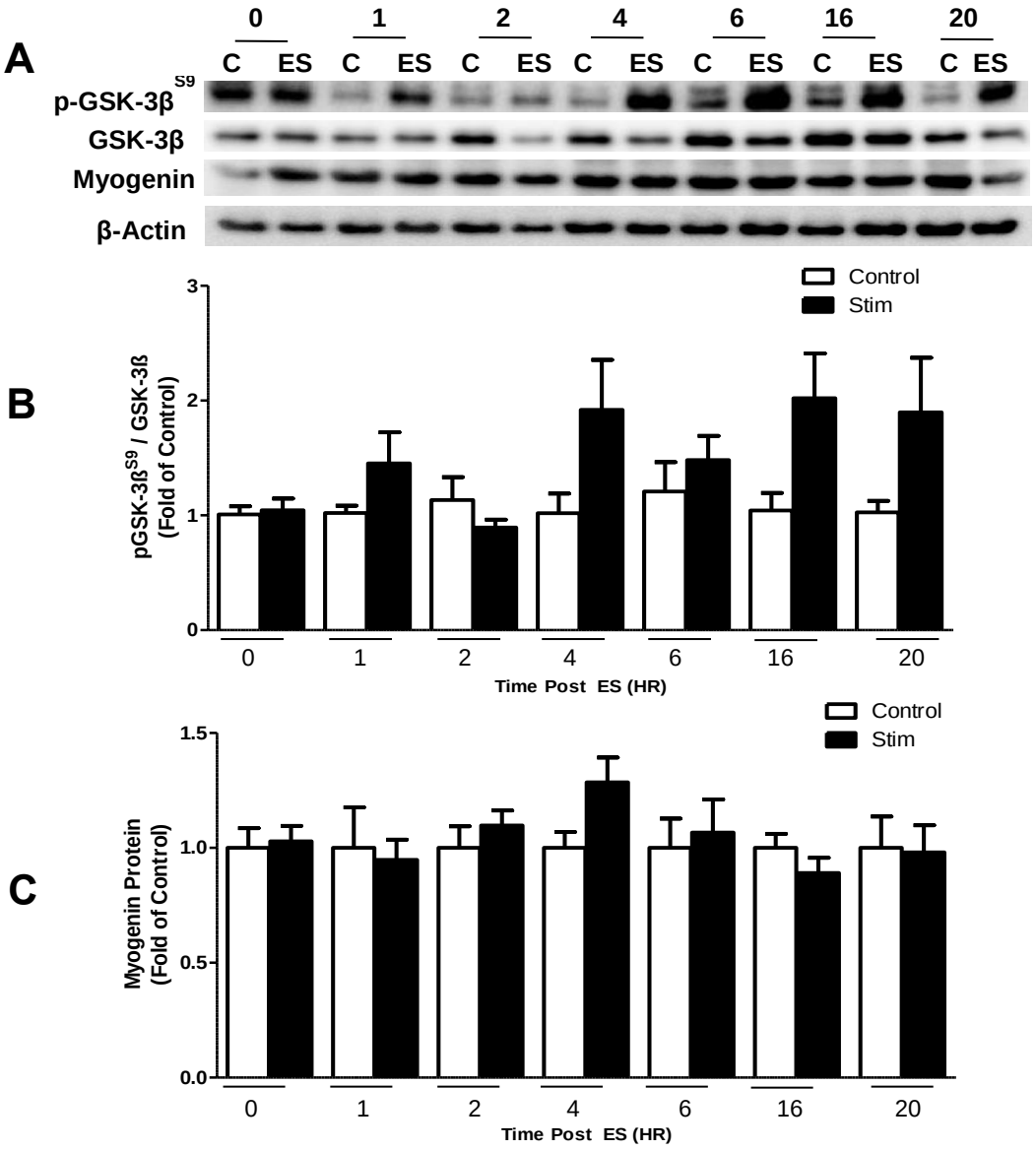

Figure 12: p-GSK-3β^{S9} and myogenin protein are unchanged following electrical stimulation (ES) in comparison to time matched control cells (C). GSK-3β was not phosphorylated more in ES cells compared C cells, although an increasing trend was evident late in the recovery period **(B)**. Myogenin protein content did not change post-ES, nor was it different than C **(C)**. All values are mean ± S.E.M.; ***** p<0.05 compared to time matched controls (n=4-6).

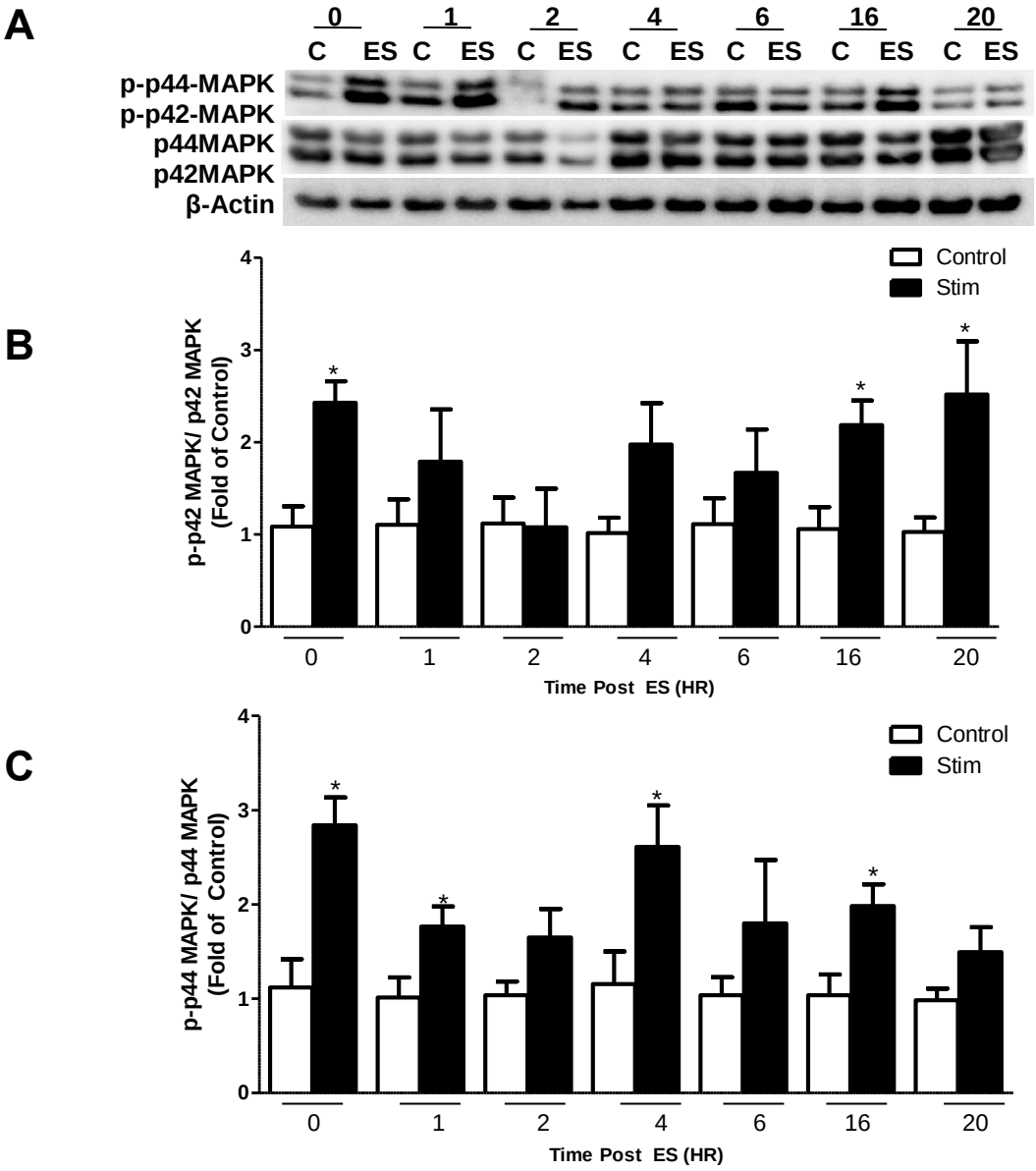

Figure 13: Electrical stimulation (ES) promoted p42 and p44- MAPK phosphorylation, although the response following ES differed. p42-MAPK phosphorylation was greater in ES than C treated myoblasts immediately following stimulation, and not-significantly greater until late in the recovery period **(B)**. p44-MAPK phosphorylation was greater in ES than C treated cells immediately following stimulation, and remained elevated until 20hours post-ES **(C)**. All values are mean ± S.E.M.; ***** $p<0.05$ compared to time matched controls (n=4-6).

Electrical stimulation may alter the differentiation capacity of proliferating C2C12 myoblasts

p38-MAPK is important in promoting differentiation through phosphorylating and subsequently activating Mef2A-D. Following ES there was a 41% reduction in total p38 protein content, and this was maintained throughout the recovery period (Figure 14A,B). Phosphorylated p38 was not detected at any time points following stimulation. (Figure 14A). MEF2A and D protein content was measured, as they are integral for the muscle differentiation process, and have the ability to be activated by p38. MEF2A protein content was significantly reduced by 40% immediately following ES. MEF2A levels began to rise, reaching control levels between 2 and 6 hours post ES. Following this, MEF2A levels declined by 57.2% less than those in controls by 20 hours post stimulation, the lowest value in the recovery period (Figure 14A,B). In contrast, Mef2D protein content is not changed in response to ES (Figure 14A,C).

$p27^{T198}$ is a stable form of p27 in response to electrical stimulation

In response to ES, there is a reduced cell count, which could be due to cell death or cell cycle arrest. Furthermore, p27 is a target of both AMPK and AKT following their activation, and thus p27 was measured to determine if it was responsible for the reduced cell count in response to ES. Immediately following ES, p27 protein levels were reduced by more than 55% compared to non-stimulated control cells (Figure 15A,B). This reduction was maintained through the entire post-ES recovery period. Given that AMPK was increased immediately following ES in comparison to control cells, we measured $p27^{T198}$, and found that its protein levels were unchanged throughout the

post-ES recovery period (Figure 15A,C). Thus, when expressed relative to total p27, the relative amount of $p27^{T198}$ was anywhere between 1.87-fold and 3.49-fold greater in ES cells than time matched controls following ES and the recovery period (Figure 15D).

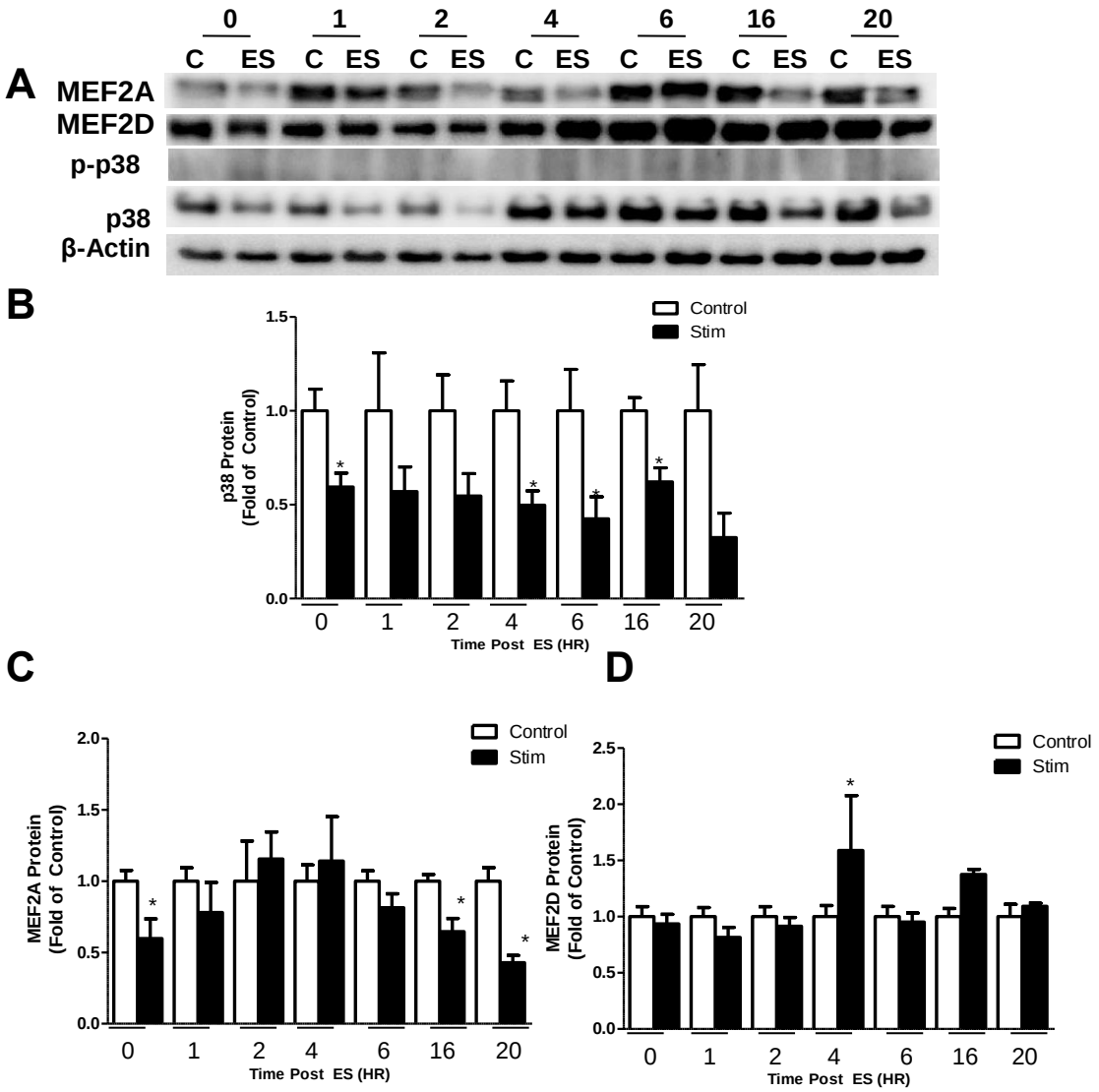

Figure 14: Electrical stimulation (ES) of proliferating C2C12 myoblasts reduced p38-MAPK and MEF2A protein content and MEF2D protein remained unchanged. p38-MAPK protein content was reduced following ES in comparison to time matched control cells **(A)**. MEF2A protein content was reduced following ES and increased to control levels throughout the recovery period followed by a decrease in comparison to control **(C)**. MEF2D protein levels in ES-cells did not change throughout the recovery period versus time matched controls **(D)** All values are mean ± S.E.M.; ***** $p<0.05$ compared to time matched controls (n=4-6).

We were surprised that total p27 decreased in cells post-ES given the large loss in cell number. To further explore the p27 response to 4 hours of ES we determined that total p27 accumulation was in the nucleus in non-stimulated cells (90%) and ES increased this to 95%, coming back to control levels of 90% 20 hours post-ES (Figure 16A,B). In contrast, majority of the $p27^{T198}$ accumulated in the cytoplasm of these cells (Figure 16A,C). However, ES caused 22% of the $p27^{T198}$ protein to be located within the nucleus, while 6 hours-post ES 39% of the protein was located in the nucleus, which amounts to an 80% increase in nuclear $p27^{T198}$ 6 hours post-ES. Nuclear levels were subsequently reduced to 26% after 20 hours (Figure 16A,B). Furthermore, to ensure the nuclear integrity of the fractioned cells was not compromised by digitonin treatment, we measured RCC1, a nuclear protein, and determined that we had only permeabilized the plasma membrane (Figure 16A). Amido black staining of the PVDF membrane was conducted to confirm that nuclear and cytoplasmic fraction compositions differed (Figure 16A).

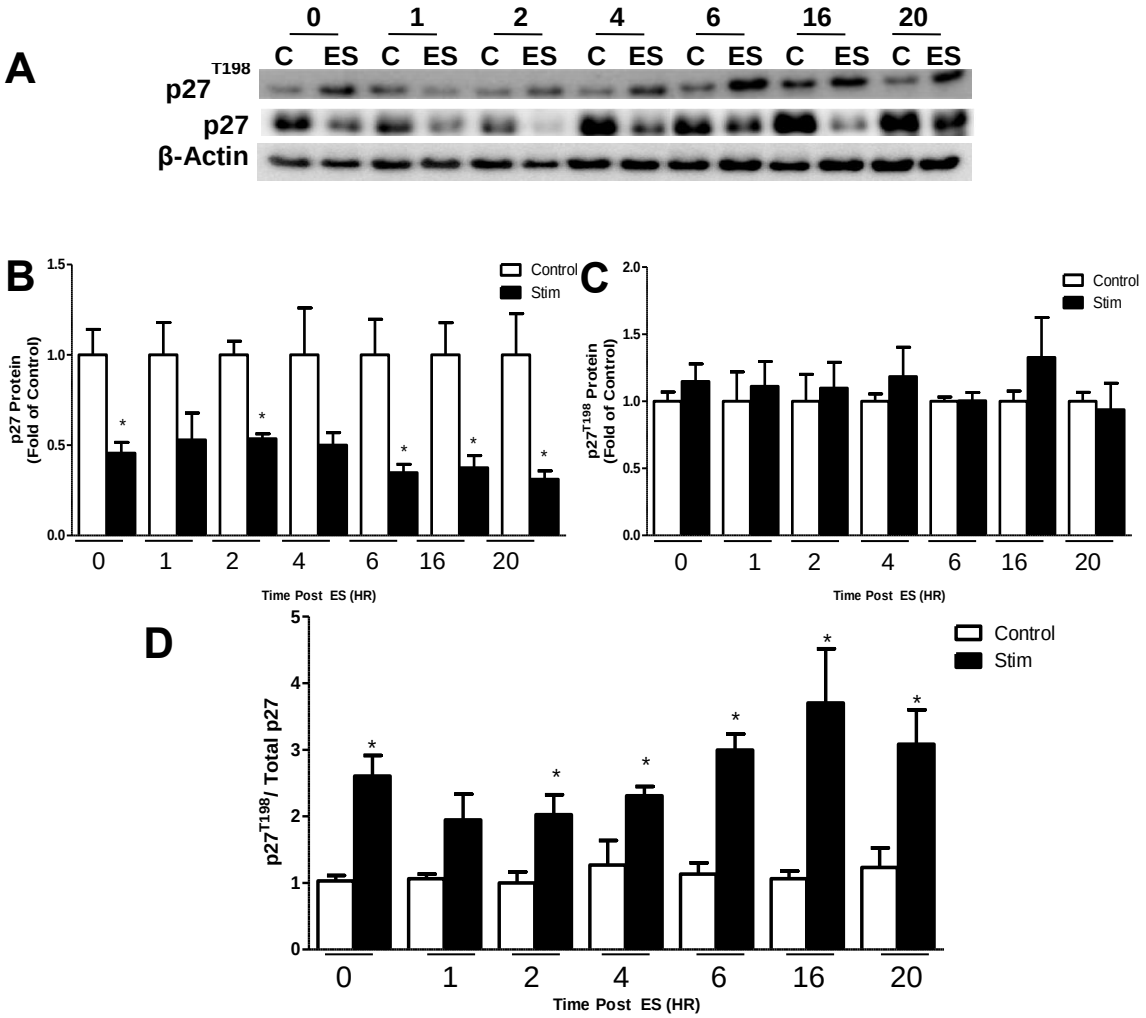

Figure 15: Electrical stimulation (ES) reduced p27 protein levels and increased the relative phosphorylation of p27on the T198 residue. p27 protein levels were reduced following stimulation in comparison to time matched control **(B)** T198 phosphorylation of p27 was unchanged following stimulation **(C)**. The relative amount of p27 that was phosphorylated on the T198 residue was greater following ES than in time matched controls **(D)**. All values are mean ± S.E.M.; ***** $p<0.05$ compared to time matched controls (n=4-6).

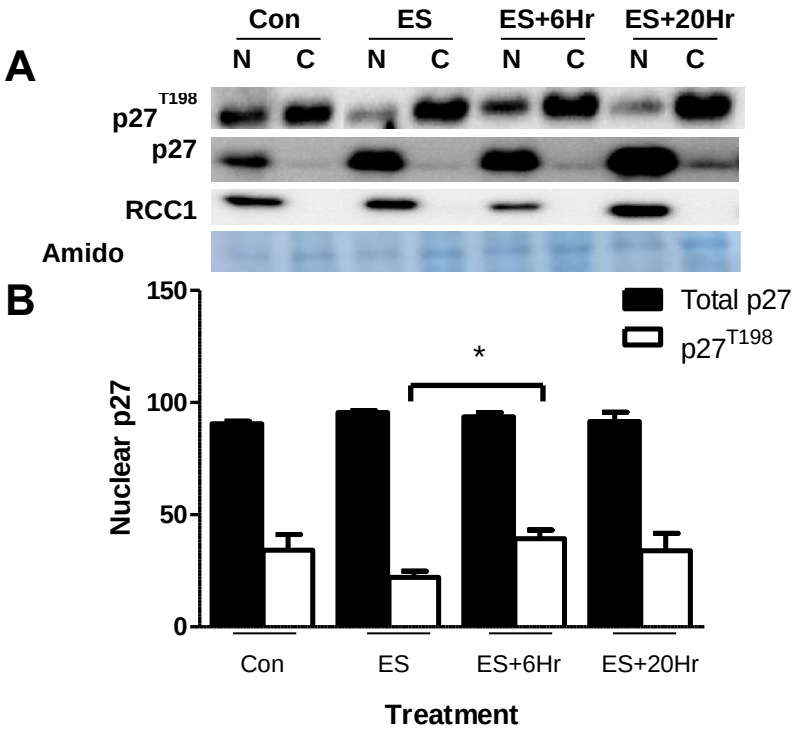

Figure 16: p27 was localized within the nucleus of proliferating C2C12 myoblasts, whereas $p27^{T198}$ was localized within the cytoplasm of proliferating C2C12 myoblasts in both control and ES conditions. Total p27 within the nucleus increased in response to ES, $p27^{T198}$ increased within the nucleus of cells in response to ES **(B)**. All values are mean ± S.E.M.; *p<0.05 (n=3).

Electrical stimulation immediately activates but does not maintain autophagy

ES led to a reduced cell count in comparison to control cells. This could be due to autophagy, which can promote cell cycle arrest and cell death. Furthermore autophagy can be positively regulated by AMPK and negatively regulated by AKT. As an indicator of autophagy we measured the conversion of LC3I to LC3II following ES, as LC3II levels are proportional to autophagosome formation and is a common marker of autophagy. Following ES there is a 2.6-fold increase in LC3II, with a peak 4.5-fold increase in LC3II evident at 2 hours post-ES (Figure 17A,B). Interestingly, this was

followed by a reduction in LC3II and a return to levels similar to those seen in non-stimulated control cells by 16 hours of recovery (Figure 17A,B).

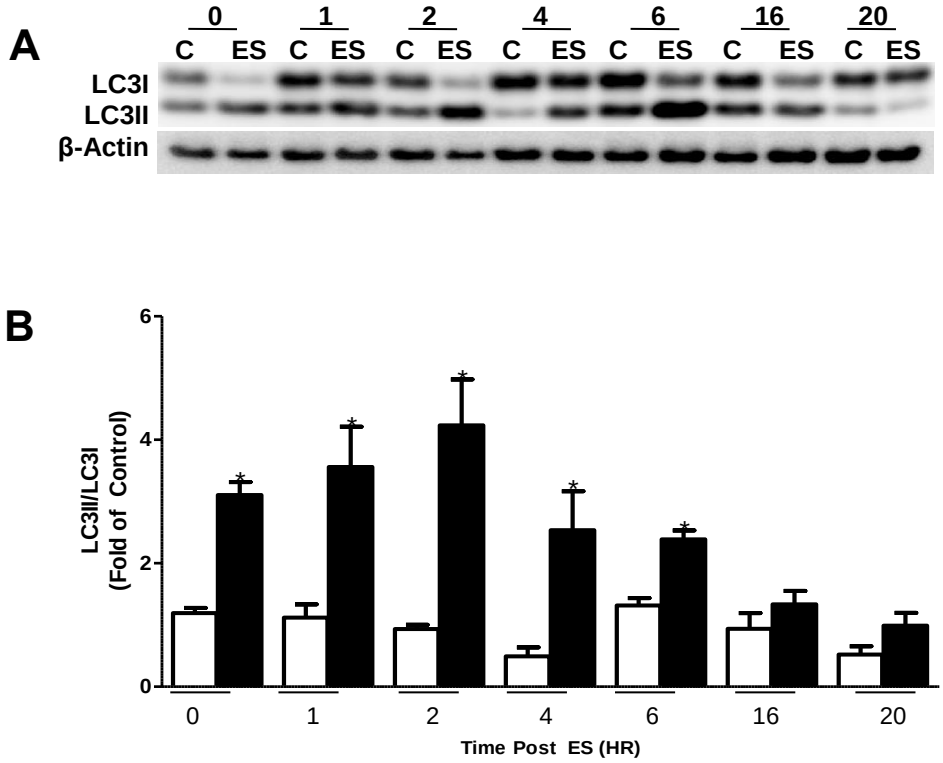

Figure 17: Immediately following stimulation there was an increase conversion of LC3I to LC3II that was normalized late in the recovery period. Stimulation increased LCII/LC3I in comparison to time matched controls immediately following stimulation and this was normalized to control levels by 16 hours post-ES **(B).** All values are mean ± S.E.M.; ***** $p<0.05$ compared to time matched controls (n=4-6).

4.5 - Discussion:

Electrical stimulation (ES) is a method commonly employed to induce intracellular changes within cells (10, 35, 41, 44). It has the ability to promote changes within C2C12 myoblasts that may alter the development of these cells into functional myotubes. It also provides insight into the importance of regulated signalling within myoblasts. No work to date has focused on the immediate early responses of proliferating myoblasts to ES. Thus the goal of this work was to investigate early signalling within these ES-cells and evaluate the effects of any changes on C2C12 proliferation.

ES of myoblasts causes a reduction in cell number. This result can be due to cell cycle exit or cell death. Previous work has shown that long term ES (3-5 days) of proliferating myoblasts promotes AMPK-mediated cell cycle exit by increasing p27 and MyoD, mediated by increases in intracellular Ca^{2+} (44). However, this reduction in cell number could also be due to increases in programmed cell death, such as that seen with prolonged autophagy (118). No increases in autophagy activity were evident after 2 and 4 days of ES (Appendix 1, Figure 20), suggesting that long term ES does not increase autophagy. However, immediately following ES the situation is very different. AMPK is activated in response to 4 hours of ES and this was accompanied by an increase in LC3II (Figure 10B and 17B), although LC3II returns to pre-ES levels during the recovery period, a response similar to AMPK. LC3II is not increasing long term, which is in contrast to what is observed in rhabdomyosarcoma (RMS) cells, which express many of the same myogenic proteins, whereby LC3II is elevated by long-term stimulation (10). This may be due to the transient upregulation in AMPK activity which

promotes the early autophagy (68, 83, 100), while the sustained AKT activation leads to the suppression of autophagy, possibly through Beclin-1 and mTOR (69, 239). Overall it appears AMPK may increase autophagy post-ES, which acts as a remodelling process within these cells, however, AKT overrides this in the long term (Appendix 1, Figure 19; 10), different from the proposed autophagy induced cell death seen in RMS cells (10).

ES activates AMPK immediately following treatment and decreases late in the recovery from ES. AMPK activation increases after 5 days of ES treatment, suggesting that AMPK activation in response to long term ES may be a cumulative response, building upon itself day by day. Furthermore, AMPK has the ability to promote cell cycle exit in C2C12 myoblasts through phosphorylation of p27 on T198 in response to ES (44), which represents a possible mechanism for the reduced number of cells following ES. Interestingly 1 bout of ES reduced total p27 protein content, which is contradictory to promotion of cell cycle withdrawal (29, 141). $p27^{T198}$ appears to be resistant to this ES-induced effect given that $p27^{T198}$ is unchanged following 4 hours of ES, which agrees with previous research on the stability of $p27^{T198}$ in response induced total p27 degradation (106, 203). We show that the majority of p27 is nuclear and yet surprisingly, $p27^{T198}$ is highly cytoplasmic (113). There appears to be an increase in nuclear total p27 immediately following stimulation and the reduction in total cell p27 suggests that there is a decline within the cytoplasmic fraction. This is also suggested by the increase in nuclear $p27^{T198}$ during the recovery phase, suggesting a possible $p27^{T198}$ translocation. This translocation may represent an important step in shutting off proliferation in C2C12 myoblasts following ES. Since ES allows p27 to bind cyclin E more readily in myoblasts (44), $p27^{T198}$ may have increased affinity for cyclins and this

phosphorylation may be highly important in cyclin/CDK inhibition and cell cycle arrest in response to ES.

Activation of AKT has been linked to the regulation of both muscle proliferation and differentiation. AKT has the ability to promote proliferation by preventing p27 nuclear import via T157 phosphorylation (122) and inhibiting FOXO transcription factors which increase the expression of p27 and reduce cyclin D levels (22, 30, 105, 133, 204, 214). However, ES-dependent AKT activation does not appear to be enhancing proliferation, as indicated by the reduced number of cells in response to long term ES (44). Rather, AKT may be exhibiting its pro-differentiation effects due to an apparent late trending increase in GSK-3β phosphorylation and thus inactivation. This may be promoting differentiation by alleviating its repression on myogenin, which is necessary for skeletal muscle fibre formation (188), although these effects on myogenin are not evident by 20 hours post-ES. Long term stimulation may result in significant increases in GSK-3β phosphorylation like those found previously in a rhabdomyosarcoma (RMS) cells (41) and promote differentiation in ES-treated proliferating myoblasts.

Active p38-MAPK is important in slowing proliferation and promoting differentiation as is it activates MEF2A and MEF2C directly (162, 262) and inhibits cyclin D indirectly (2, 116, 226). Interestingly, ES induced a reduction in p38 protein content, while phosphorylated p38 was undetected by western blot. Since p38 is activated upon mitogen-depletion to promote differentiation (23, 245), the lack of p38 phosphorylation suggests that differentiation was not being properly being induced by ES. Furthermore, the reduction in total p38 in response to ES suggests a reduction in the pathway capacity and may have negative ramifications on the ability of these cells to differentiate

by decreasing p38 activation of MEF2s (8). MEF2A has been shown to be essential in myoblast differentiation and muscle regeneration, whereas MEF2 B, C, and D are not (47, 207). The decline we see in MEF2A in response to ES may further limit terminal differentiation. Thus, although ES appears to be ceasing proliferation, it may limit the differentiation capacity of the myoblasts and may explain why long term ES appears to induce premature differentiation (44)

Our results indicate that ES causes multiple changes in intracellular signalling within proliferating C2C12 myoblasts, promoting premature cell cycle withdrawal and autophagy mediated cellular remodelling. This may have ramifications in muscle maintenance, whereby activation of a satellite cell prior to or during cell fusion with existing muscle fibres, may impair proper repair of injury or myonuclei renewal.

5.0 – Overall Conclusions:

Satellite cells serve as proliferative-capable muscle precursor cells that give rise to myoblasts following their activation and exist on the periphery of myofibres. Following muscle injury there is an activation of satellite cells, which proliferate and subsequently differentiate, donating their nucleus to the injured muscle, a vital step in regeneration. Numerous approaches exist to explore the molecular mechanisms involved in the muscle regeneration process. We employee an electrical stimulation (ES) model as a method of altering the signalling within proliferating C2C12 myoblasts to further understand the mechanisms involved in myoregeneration. We can then investigate the early signalling response of proliferating myoblasts following exposure to ES and better understand how electrical stimulation of myoblasts affects regenerative capacity.

There is a reduction in proliferation of C2C12 myoblasts following as little as one 4 hour bout of ES, as evident by the reduction in cell numbers. This was accompanied by long term morphological changes, whereby myoblasts align in a fashion that suggested that like they were preparing to fuse and differentiate (44). This cell cycle arrest may occur prior to the G1/S transition due to increases in p27 binding to cyclin E (44). My thesis was designed to examine the immediate response of proliferating C2C12 myoblasts to 1 bout of ES in aims of understanding the long term effects that are promoted by ES-treatment.

The AMPK, AKT and MAPK signalling pathways have diverse roles in the processes of proliferation and differentiation, and all have been shown to affect the cell cycle and myogenesis. They have observed differential effects by acting on proliferation

in a sub-confluent state and differentiation following confluence and mitogen depletion (30, 79, 103, 245). The alterations in the activation of these proteins have the ability to promote changes in the developmental program within these myoblasts. Thus, the changes seen in response to ES have the potential to affect both proliferation and differentiation within these myoblasts. My observations suggest that the forced activation of these proteins by ES act in a manner to promote cell survival and prevent cell division, and it appears that ES decrease the differentiation capacity of these cells.

ERK1 and 2 respond differently to ES, indicating that although closely related, the ERKs respond uniquely to ES. Interestingly, ERK1/2 can promote both proliferation and differentiation, however its inactivation is necessary for the transition from proliferation to differentiation (1, 64, 78, 88, 144, 163, 222). Thus ES may be prematurely activating ERK1/2 and promoting differentiation or ES may be inducing changes in ERK1/2 localization from the nucleus where it induces proliferation, to the cytoplasm where it is associated with differentiation (136). Whether this shift in ERK1/2 localization occurs warrants further investigation.

It appears that ES is inducing a targeted degradation of specific proteins within the cell. Furthermore, the phosphorylation of AMPK, AKT and p27 seems to prevent their breakdown, suggesting these phosphorylations are important in stability of the protein, agreeing with previous work (48, 106, 203). Additionally, total p38 and MEF2A are reduced, and phosphorylation of these proteins was not detected or measured. Thus, no phosphorylation induced stability effects can be inferred. This raises the question of whether the relative or absolute activation of these proteins is important for

intracellular signalling. My work suggests that the relative activation is important in terms of AKT and p27, as the downstream targets are activated in response to ES.

In response to low energy conditions and cellular stress, autophagy can be activated in aims of returning cellular energy balance. It does so through organelle breakdown and recycling intracellular components, and in further promotes cell cycle arrest (124). Autophagy functions in cellular remodelling, such as with the mitochondria and mitophagy, in response to exercise-induced stress and disuse/denervation induced stress (26, 87, 92, 230, 231). This promotes destruction of dysfunctional mitochondria and it may be similar to what we observe, albeit at an individual cellular level. Excessive degradation of intracellular components by autophagy has been linked to cell death (118). In response to ES, there is an immediate increase in autophagy followed by a normalization to control levels. Thus deficient cells may undergo autophagy, indicating that autophagy may be promoting cellular “pruning” in response to ES. This immediate response in C2C12 cells is similar to what is observed following long term ES in rhabdomyosarcoma cells (10). However, long term-ES does not induce prolonged activation of autophagy, suggesting that despite both cell types expressing muscle-specific proteins, their adaptation to ES is very different.

The observed reduction in cell number, lack of long term autophagy, and the increased stability of $p27^{T198}$ following ES lead to further investigation of p27 as a regulator of ES-induced cell cycle arrest. Specifically, I wanted to investigate where p27 is localized, and if the reductions in p27 were localization specific in response to ES. Reductions in total p27 seem to be in the nuclear fraction of the myoblasts, whereas $p27^{T198}$ accumulates within the nuclei and cytoplasm of these cells. Thus, it seems that

$p27^{T198}$ drives the cell cycle arrest in ES-myoblasts, potentially due to enhanced nuclear import of $p27^{T198}$ or greater binding affinity for the cyclins to promote cell cycle arrest (Figure 18), similar to the finding that p27 binds cyclin E following long-term ES promoting cell cycle arrest.

Overall the change in observable characteristic elicited by ES within proliferating myoblasts underlies the importance of coordinated signalling within C2C12 myoblasts in terms of myogenesis and muscle regeneration. ES may in fact be a hindrance to effective therapy following muscle damage, as it may limit the proliferative and differentiation capacity of the activated satellite cells, thereby preventing the necessary donation of muscle progenitors to the damaged muscle fibre. However, this does not leave out the possibility of using ES as method of aiding in the differentiation process, which would require proper timing in response to muscle damage. Stimulation too early may hinder proliferation, whereas if timed appropriately may enhance differentiation. Further experiments are required to determine the use of ES as a possible therapy following muscle damage and in response to muscle wasting or myopathies.

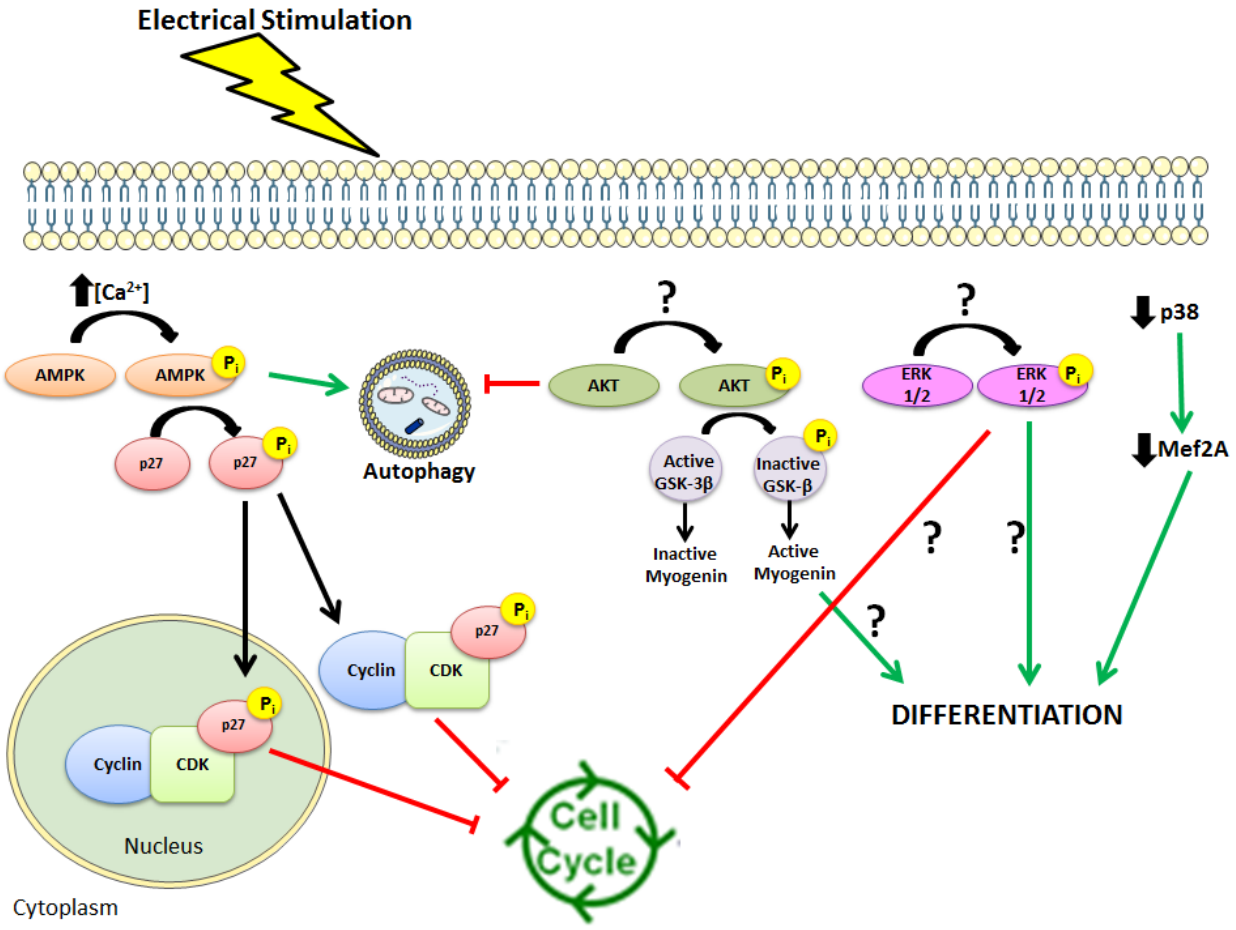

Figure 18: Current working model based on data and observations collected. Electrical stimulation (ES) promotes intracellular changes within proliferating C2C12 myoblasts. Cell cycle arrest is in part mediated by p27. Autophagy is promoted early in the post-ES recovery pervious, but autophagy is not withheld, indicating a remodelling of intracellular components. Differentiation may be limited following ES due to reductions in p38, Mef2A, and a disconnect between AKT, GSK-3β and myogenin.

6.0 - Limitation and Future Directions:

6.1 – Limitations:

The current work utilized electrical stimulation (ES) as a method of the altering signalling within proliferating C2C12 myoblasts. This *in-vitro* work provides plenty of insight into the importance of proper signalling within the development of musculature and how dysregulation in signalling affects the normal myogenic program, however *in-vitro* work is not always reproducible in an animal model, and thus the results would need to be further investigated using an animal model in order to gain insight into the true physiological ramifications of ES on skeletal muscle regeneration. Furthermore, I use proliferating C2C12 myoblasts as a model cell of post-activated satellite cells, however these cells are not satellite cells and despite the similarities, may not respond to ES the exact same way that satellite cells do. To investigate the response of ES on satellite cells, primary satellite cells would need to be extracted from intact and exposed to ES. These limitations do not indicate that using myoblasts in an *in-vitro* model are not appropriate for studying the effects of ES and altered signalling on muscle regeneration. However, they pose a constraint on the ability to generalize my findings in a physiological setting.

6.2 - Future Directions:

The current work along with previous work answered many of the questions proposed in this study. However, it does create new questions to be answered, requiring further investigation.

6.2.1: Cause of Reduced Cell Count

A reduction in the number of myoblasts is evident following ES, yet the reasons for this observation are not fully understood. Further experiments are necessary to determine whether this reduced cell count in response to ES is mediated through the onset of cell cycle arrest, autophagy mediated cell death or increases in apoptosis. Apoptotic markers were not measured in the current work. Next steps would include measurement of markers such as Bcl-2, AIF, caspase3,6 or,7 or BH3 in response to ES. This would provide insight into if apoptosis is occurring and whether it is through a caspase-dependent or independent mechanism. Furthermore, only LC3 was used as a marker of autophagy. But, to gain insight into whether autophagy is functionally increased within these cells, and if it is an immediate response to ES, more markers of autophagy must be measured. Examples of this would be Beclin-1, ATG3, ATG7, or any complexes formed throughout autophagy. Furthermore, lysosome activity could be measured to see if there is flux through the autophagy pathway.

The results indicate that the reduction in cell count in response to ES is likely due to cell cycle effects of ES on myoblasts. In RMS cells ES induced a G2/M cell cycle arrest and long term autophagy, and ES induced G1/M cell cycle arrest was suggested from previous work done in ES-myoblasts. FACS analysis of ES-myoblasts would determine where majority of the cells accumulate within the cell cycle. FACS could further direct the research to investigating cycle-specific events that promote cell cycle arrest. Furthermore it could provide insight as to whether autophagy or apoptosis is taking place depending on if the cells are arrested in G1/S or G2/M. Although ES increased the specific levels of $p27^{T198}$ and it is localized within both the nuclei and

cytoplasm of these cells. Other cell cycle inhibitors may also be promoting cell cycle arrest in response to ES, such as p21 and p57, and their protein content and activity should be measured to determine the role they play in ES-mediated proliferative declines. p21 might be of prime interest due to its direct connection with MyoD within muscle and ES has been shown to increase MyoD expression in proliferating C2C12 myoblasts (44). Furthermore, immunoprecipitation of both total and $p27^{T198}$ with various cyclins in whole cell extracts and nuclear and cytoplasmic fractions will determine the interactions and cell cycle ramifications of $p27^{T198}$ within the cytoplasm and nucleus of ES-myoblasts.

6.2.2 – Ramifications of Intracellular Signalling:

Widespread intracellular changes were observed after as little as a single 4 hour bout of ES, illustrating ES as a powerful tool for inducing phenotypic changes. However the downstream effects of these changes have yet to be investigated, and warrant further investigation in order to understand the cellular events that take place in response to ES and its applications within myogenesis. It is unknown at the present time if the AMPK activation is AMP:ATP driven, or Ca^{2+} driven. However, previous work does indicate it is Ca^{2+} mediated and chelation of $[Ca^{2+}]_{IC}$ will provide necessary insight into what is responsible for AMPK activation. Moreover, inactivation of AMPK with Compound C could uncover a mechanism by which AMPK alters myoblast proliferative capacity in response to ES.

AKT activation is thought to prevent autophagy and promote cell growth and division. However, this is contradictory to what we observed after ES, thus the

consequence of AKT activation following ES could be further investigated through AKT inhibition, by inhibiting its receptors/activators, using siRNA, or pharmacological inhibition. This could provide insight into the role of AKT activation during the C2C12 myoblast response to ES. These studies would also allow investigation into the dysregulation of the AKT-GSK3β-myogenin axis in response to ES.

The mitogen sensitive protein kinases (MAPKs) play an integral role in both proliferation and differentiation of myoblasts. I show that ERK1/2 are activated in response to ES but the ramifications of this activation remain unknown at the present time. The diverse roles of these MAPKs within cells were not investigated in the current study, although measurements of downstream targets of ERK1/2 could provide insight into the roles of p42 and p44 in response to ES. Furthermore, like AKT, ERK1/2 could be pharmacologically inhibited or silenced using siRNA's to investigate the role it plays in the ES-response. Cellular localization assays of p42 and p44 would further determine the role their location plays in the proliferation and differentiation of myoblasts and the consequence of its potential localization change following ES treatment.

6.2.3 – Differentiation Capacity of ES Myoblasts:

The differentiation capacity of myoblasts following ES appears to be compromised. To evaluate this, ES-treated cells could be exposed to differentiation conditions and any effects on the speed or completeness of differentiation could be measured. Such measures could be a fusion index or MHC in comparison to control cells. The dramatic reductions in p38 and Mef2A could have negative effects on the

differentiation capacity of these cells, although this work could answer the question if these cells are capable of differentiation.

Appendix 1 – Supplementary Data:

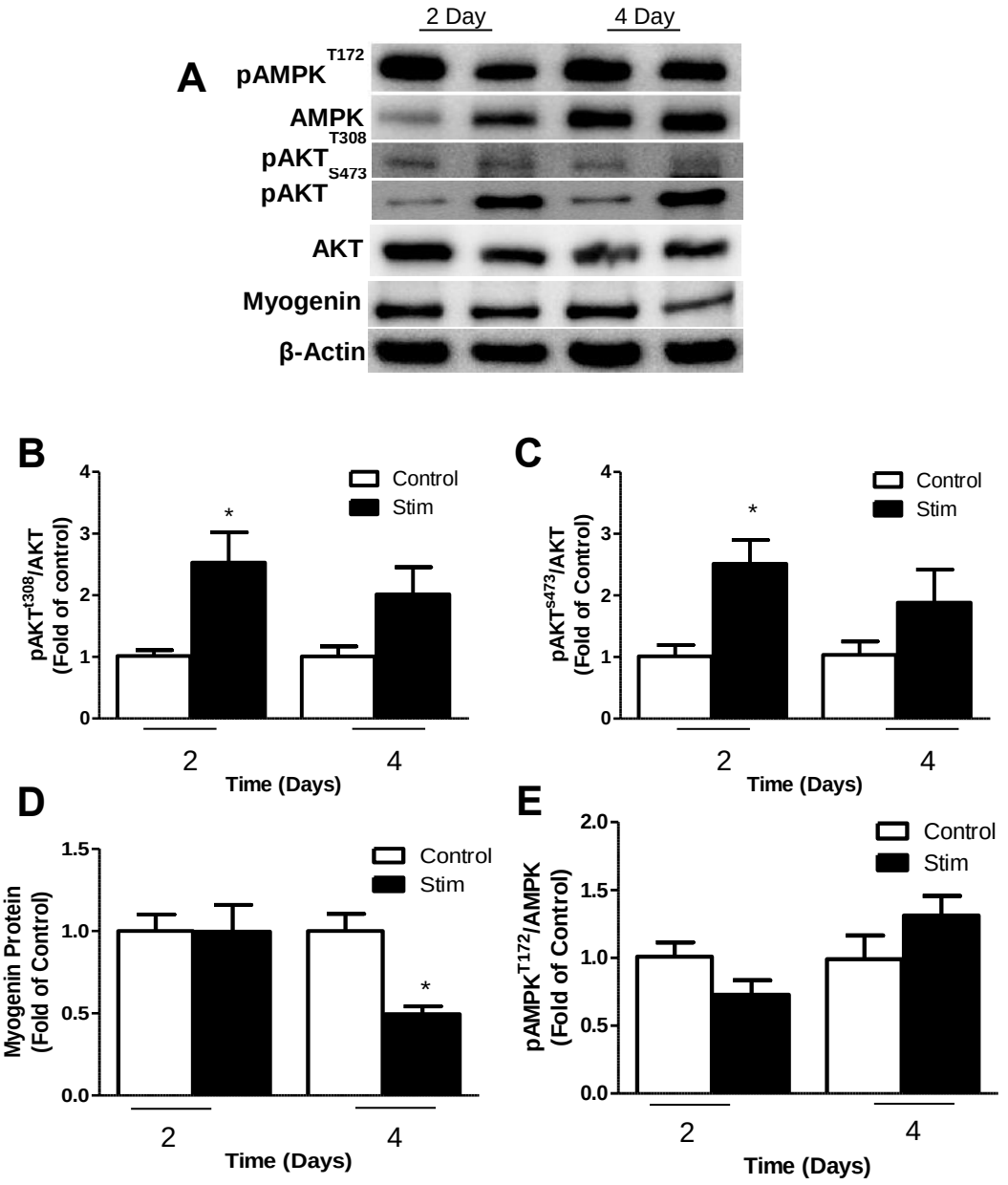

Figure 19: 2 and 4 days of electrical stimulation (ES) promoted the activation of AKT, reductions in myogenin protein content and no activation of AMPK in proliferating C2C12 myoblasts **(B)** AKT was phosphorylated on the T308 residue following 2 days but not 4 days of ES in comparison to control. **(C)** AKT was phosphorylated on the S473 residue following 2 days but not 4 days of ES in comparison to control. **(D)** Myogenin protein content was reduced by 4 days of ES in comparison to control cells. **(E)** AMPK was not phosphorylated following 2 or 4 days of ES. β-Actin was used as a loading control and all values are mean ± S.E.M.; ***** $p<0.05$ compared to time matched controls (n=4-6).

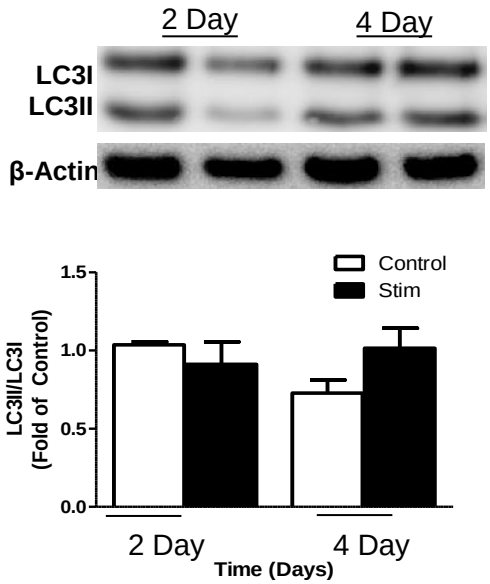

Figure 20: LC3II/LCI protein levels in C2C12 myoblasts was unchanged in response to ES. **(B)** ES and C cells show no difference in the proportion of LC3II to LC3I. β-Actin was used as a loading control and all values are mean ± S.E.M.; ***** $p<0.05$ compared to time matched controls (n=4-5).

AKT is an important signalling molecule within cells, and has the ability to regulate proliferation and differentiation of C2C12 myoblasts. I demonstrate that in response to a single bout of ES, AKT is activated (Figure 11), and the same is true with longer term-ES (Figure 19 A-C). This may have profound effects within these proliferating myoblasts, where it may promote activation GSK-3β and its targets, such as myogenin and cyclin D, leading to cell cycle arrest and the onset of the differentiation program. However, long term stimulation was shown to promote cell cycle arrest, while limiting the differentiation capacity of the cells (44).

Myogenin is a downstream target of AKT and AKT mediated phosphorylation of GSK-3β alleviates the repression GSK-3β puts on myogenin. In response to ES, there

is no initial change in myogenin protein levels (Figure 12), although there is a drastic reduction in myogenin (0.50 ± 0.11) following 4 days of ES (Figure 19), indicating there may be a disconnect between AKT, GSK-3β and myogenin as a result of ES with AKT potentially promoting cell survival rather than differentiation.

It was previously demonstrated that following 5 days of ES there is an increase in AMPK activation via its phosphorylation on T172. In the current study I was unable to show AMPK activation after 2 or 4 days (Figure 19D), but there was still a decreased cell number in response to ES. Immediately following ES there is an increase in AMPK activation (Figure 10), which suggests that AMPK may be an immediate responder to ES, and that long term stimulation promotes an increase in its activation that is built up over time. This may lead to increases in $p27^{T198}$, and alter the p27 subcellular localization (Figure 16), thereby causing an increase in association with cyclin E, and promoting cell cycle exit. Furthermore, AMPK is an activator of autophagy, and may be promoting the short term increase in LC3II/LC3I we see immediately in response to ES.

LC3 is an important part of the autophagic pathway. The lipidation of LC3I to LC3II indicates it is active and may cause an increase in autophagy. While one bout of ES increases LC3I conversion to LC3II, during the longer term (2 and 4 days), ES does not (Figure 20). This differs from what was previously found in rhabdomyosarcoma cells, in which ES elicited an increase in LC3II following long term stimulation, and was thought to act as a cell death pathway, rather than a cell remodelling pathway as thought to be the case in these myoblasts (10).

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