Examining the Role of Apoptotic Cell Signalling and Mitochondrial Fission During Skeletal Muscle Differentiation

by

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Author's Declaration

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

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Abstract

Cellular maturation (differentiation) and cell death (apoptosis) are two vital processes shared by virtually all mammalian cells types. Although these two events have disparate outcomes, recent evidence indicates their execution may involve similar cellular mechanisms. Considered the primary effectors of apoptosis, a family of proteolytic enzymes known as caspases become activated in response to upstream apoptotic signalling, and are responsible for cleavage of structural and regulatory proteins, nuclear degradation and DNA fragmentation, and cell blebbing. While these enzymes have a well-defined role in death, current research suggests their activity is necessary during the differentiation of several cell types including skeletal muscle. However, it is currently unknown how this pro-apoptotic environment is regulated to promote differentiation. A long known mediator of apoptotic signalling, the mitochondria, has recently been shown to affect apoptosis through changes to its morphology. Mitochondrial division (fission) and fusion are necessary for maintaining normal cellular function, although fission contributes to apoptotic signalling. In this study, we examined the mechanisms which lead to caspase activation during skeletal muscle differentiation, and determined the importance of mitochondrial fission to this process. It was hypothesized that typical mitochondrial-mediated apoptotic signalling would be responsible for activating caspases during myogenesis, partly due to increased fission. C2C12 mouse skeletal myoblasts maintained in culture were induced to differentiate by switching to low growth-factor media and collected at various time points during the differentiation process. Activity levels of caspases-2 and -3 transiently increased 51% and 2.5-fold, respectively, 1.5 days after inducing differentiation ($p<0.05$). No changes were observed in the activity levels of caspases-8 and -9. Although whole-cell levels of Bax and PUMA increased 16% and 21% ($p<0.05$), respectively, prior to the spike in caspase activity, levels of mitochondrial-Bax were matched by Bcl-2, resulting in no change to the mitochondrial Bax:Bcl-2 ratio early during differentiation. This ratio indicates the susceptibility of the mitochondria to release pro-apoptotic factors, and was associated with decreased cytosolic levels of Smac and cytochrome c by 63% and 75%, respectively, early during differentiation ($p<0.05$). Levels of the anti-apoptotic proteins Bcl-2 and ARC increased ($p<0.05$) as caspase activity diminished, possibly supporting their role in ensuring temporary caspase activation. Pharmacological inhibition of caspase-3 resulted in reduced differentiation as indicated by decreased myotube development and cell fusion events. These morphological changes were associated with decreased protein expression levels of the myogenic transcription factor myogenin ($p<0.05$), and the mature-muscle marker myosin ($p<0.05$). Likewise, chemical inhibition of caspase-2 activity impaired myotube development, cell fusion, as well as expression of myogenin ($p<0.05$) and myosin ($p<0.05$) similar to the inhibition of caspase-3. Finally, reducing mitochondrial fission with a chemical inhibitor of Drp1 function (mdivi-1) also prevented myotube development, resulting in undetectable levels of myosin expression and a 94% drop in cell fusion events. However, these effects were not due to decreased caspase activation. In contrast to our hypothesis, these results support the notion that mitochondrial apoptotic signalling is likely not responsible for inducing caspase activity during myogenesis. Furthermore, we report that mitochondrial fission is necessary for proper skeletal muscle differentiation, likely through its contribution to mitochondrial network morphological changes associated with myotube formation.

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Introduction

Cellular Specialization

A healthy, functional organism is dependent on the ability of its cells to perform a multitude of extremely varied functions. As such, cell populations have evolved which are tailor-made to serve very specific purposes. Since the vast numbers of different cell types are derived from the same two sex cells, a process allowing for efficient cellular transformation has also evolved: this is referred to as differentiation (1-3). While the exact structural/biochemical changes which occur during this process are diverse across cell types, it is generally considered that differentiation is a process wherein cellular alterations result in phenotypic maturation that is advantageous for the organism as a whole (3). In many cells, such as keratinocytes, erythrocytes, and monocytes, differentiation occurs in a population of precursor cells present in the adult organism (reviewed in 2). These cells have a high rate of turnover, and the organism's ability to maintain a healthy number of mature/differentiated cells is dependent on the maintenance of the undifferentiated precursor population. For other cells, such as lens epithelial cells, neurons, and muscle cells, differentiation occurs during embryogenesis, with minimal turnover of the mature, functional cells during adulthood (2).

Myogenic Determination

The cells comprising mature muscle are highly specialized, unique for their ability to contract and therefore produce movement. Specifically, mature skeletal muscle cells are characterized as large, multinucleated fibers with extensive contractile apparatus composed of overlapping repeats of actin and myosin proteins. Differentiation of skeletal muscle occurs during embryonic development, whereby single-nucleated myoblasts withdraw from the cell cycle and fuse to form multinucleated myotubes (4, 5). During embryogenesis, myogenic precursor cells first become evident in the dermomyotome, a somitic structure of mesodermal origin containing a mixture of myogenic and dermal progenitors from which the myotome develops (6, 7). At this stage, appearance of the paired box transcription factors PAX3 and PAX7 is considered to identify cells as myoblasts, as their activation induces cells to follow a myogenic lineage (7-9). These two transcription factors are important regulators of skeletal muscle development and are upstream of myogenic genes in skeletal muscles (10-13). Mice with PAX3 mutations lack limb muscles although trunk skeletal muscle development still takes place (14, 15). Conversely, mice without PAX7 still express skeletal muscle markers in a normal spacial pattern within the developing myotome, although these animals die shortly after birth due to a defect in neural crest formation (16). These studies suggest a level of functional redundancy exists between these two myogenic regulators. However, in mice lacking both PAX3 and PAX7, major defects in skeletal muscle formation occur, although myoblasts are still generated via a PAX-independent mechanism (10). Once formed, myoblasts undergo a period of extensive proliferation, resulting in somite production that extends dorsally along the embryo. Signals from the adjacent developing notochord and neural tube progressively instruct these dividing myoblasts to differentiate by exiting cell cycle and fusing with each other, producing structures known as myotubes (4).

Skeletal Muscle Differentiation

Myotubes begin to develop characteristics of mature skeletal muscle, such as production of myosin and formation of myofibrils, development of the sarco-endoplasmic reticulum (SR), and gain the ability to contract (17, 18). In response to the combined effects of PAX3/7 and other molecular signals from the surrounding developing neural tube (Shh, WNT, BMP, etc.), a specific family of myogenic transcription factors become activated (4). These myogenic regulatory factors (MRFs), most notably those of the MyoD family of skeletal muscle specific, basic helix-loop-helix (bHLH) transcription factors, are known to control and initiate skeletal muscle differentiation (19-21). The myogenic bHLH proteins bind to DNA as heterodimers with other bHLH factors called E-proteins (21). Targeted activation of E-proteins is necessary for expression of several skeletal muscle-specific genes such as muscle creatine kinase (MCK), myosin light chain (MLC), desmin, and the acetylcholine receptor (AChR) (18). When expressed in several non-myogenic cell types, bHLH transcription factors initiate the skeletal muscle differentiation program (22-24). The four main members of the MRF family control skeletal myogenesis in a temporally-dependent manner. MyoD and Myf-5 are present in proliferating, undifferentiated myogenic cells, myogenin expression begins at the induction of differentiation, and MRF-4 is found in mature skeletal muscle (5). With appropriate environmental stimulation, MyoD and Myf-5 activate the myocyte enhancement factor (MEF) family of transcription factors, which are necessary to initiate transcription of effector MRFs (myogenin and MRF-4), as well as other muscle-specific genes (5, 18 (Figure 1)). Because myogenin can reciprocally activate MEF2, a positive feedback regulatory loop ensures that high levels of both MEF2 and myogenin will be maintained in differentiated skeletal muscle (18). In this way, myogenin/MRF-4 expression and coincident skeletal muscle differentiation exist down-stream in a regulatory cascade from MyoD and Myf-5 activation (although this traditional paradigm has come into question (10). The generation of specific MRF knock-out mice has provided insight into this hierarchal relationship. Myf-5 deficient mice undergo normal skeletal muscle development but die before birth due to severe rib defects (25). Interestingly, introduction of the myogenin construct into the Myf-5 locus of these animals results in healthy offspring but does not fully compensate for the absence of Myf-5 during skeletal muscle differentiation (5, 26). Mice lacking

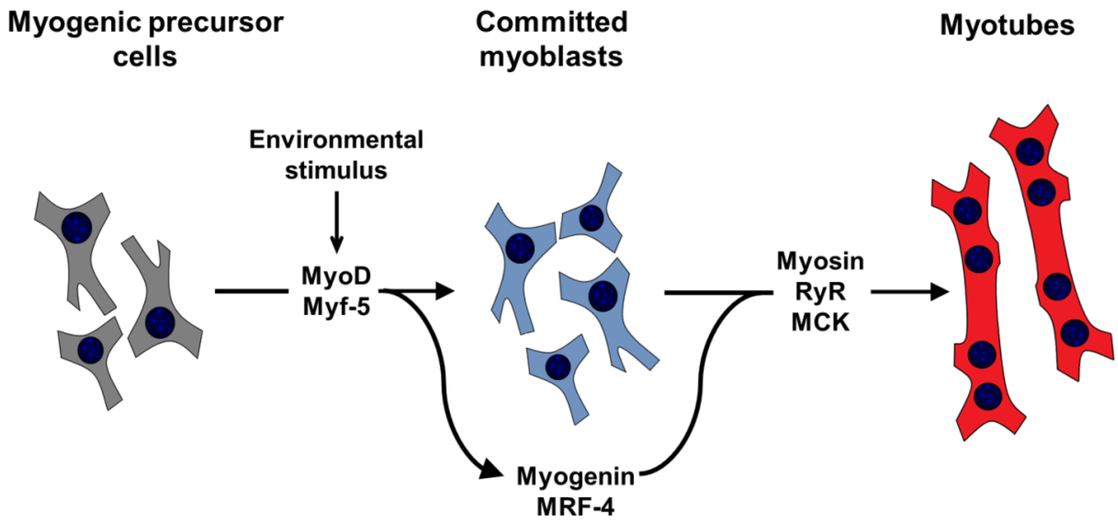


Figure 1: MRF control of skeletal muscle differentiation. During development, signals from the neural tube initiate expression of MyoD and Myf-5 in a population of cells with myogenic potential, committing them to become muscle. This leads to activation of secondary MRFs myogenin and MRF-4, which are responsible and required for transcription of mature skeletal muscle-related genes.

MyoD display an apparently normal skeletal muscle phenotype but with a four-fold increase in Myf-5 expression, suggesting a redundancy in the activity of these two initiator MRFs (27). However, mice without both Myf-5 and MyoD die at birth and are absent of any myoblasts and skeletal muscle development (28). This phenomenon has been shown occur through alterations to MRF-4, as its replacement in Myf-5/MyoD double knock-outs actually restores the presence of skeletal muscle (29). Myogenin knock-out mice possess a normal number of myoblasts but die during fetal development due to a complete absence of myotube production (30). Finally, deletion of MRF-4 results in viable mice with apparently normal skeletal muscles but a four-fold increase in myogenin expression (31, 32).

Because MyoD and Myf-5 are found in myoblasts, there must be mechanisms which inactivate their myogenic functions in proliferating cells. The inhibitors of DNA binding/differentiation (Id) family of proteins are able to inhibit MRF myogenic activity through their non-basic HLH domains (33, 34). As Id levels fall and are redistributed during differentiation, E-proteins E12 and/or E47 are free to form functionally active heterodimers with MyoD, which promote the expression of muscle-specific proteins, such as MCK, by binding to their gene promoter regions (33, 34). The protein Twist has been shown to similarly segregate E-proteins, preventing MRF/MEF-DNA binding, as well as inhibit MyoD activity through direct protein-protein interaction (35, 36). Similar to Id proteins, Twist levels decrease upon the induction of differentiation, allowing pro-myogenic MRF-DNA binding. MyoD is also negatively regulated in myoblasts by Mist1, another bHLH factor, resulting in heterodimers which do not bind to E-box-containing muscle-specific promoter regions (37). Finally, the TGF-β myostatin prevents differentiation by inhibiting both MyoD activity and expression (38, 39). Additionally, as myotubes have withdrawn from cell cycle, there must also be a connection between regulation of these transcription factors and cell cycle obstruction. Hypophosphorylated retinoblastoma protein (pRb) promotes cell cycle arrest at the G1-S phase by associating with MyoD (40, 41). Induction of differentiation results in up-regulation of the cell cycle inhibitors p21 and p16, and the gene encoding p21 is activated by MyoD (40, 42). Furthermore, cyclin D1 and Cdk4, cell cycle checkpoints at the G1-S transition, are able to inhibit MyoD activity and subsequent activation of myogenic genes (43, 44).

These molecular signalling cascades result in very specific morphological and biochemical changes, eventually generating mature skeletal muscle fibers. Although muscle is a very unique cell type, many other differentiation processes are characterized by noticeable alterations to cell morphology. In fact, the initial connection between cellular differentiation and apoptosis stemmed from the observation that for keratinocytes, lens epithelial cells, and erythrocytes, differentiation involves complete removal of the nucleus; an occurrence normally associated with apoptosis (45). A number of phenotypic alterations typical of apoptosis also occur during the differentiation of skeletal muscle. Cytoskeletal filaments reorganize during myoblast fusion, a phenomenon which also happens during the packaging of apoptotic cells (46). Second, activity of matrix metalloproteinases is required for membrane fusion associated with both differentiation and apoptosis (47, 48). Lastly, the exposure of phosphatidylserine residues to the extracellular surface typical of apoptotic cells is an integral component of cell fusion during myotube formation (49).

Typical Apoptotic Signalling Mechanisms

Because of these similarities, it was hypothesized that the execution of differentiation and apoptosis could involve similar molecular signalling mechanisms (50). The class of proteins which display the most promising biochemical link between these two divergent cellular processes is that of the caspases. Caspases are a family of proteolytic enzymes with structural homology that cleave specific substrates between cysteine and aspartic acid residues. Their activation usually represents the next-to-last step in several cell death signalling pathways resulting in apoptosis (51, 52). Caspases are generally separated into two broad categories: initiator and effector. Both classes exist as inactive zymogens (procaspases), and are activated by proteolytic cleavage, removing their pro-domain and leaving a truncated, enzymatically active form (53). Initiator caspases, such as caspases-8 and -9, are typically activated on large, enzyme-specifc, multi-subunit scaffold platforms (53). The effector class, including caspases-3, -6, and -7, are activated by initiator caspases and are responsible for the cleavage of >300 cellular substrates (54). Cleavage of these numerous substrates results in the cellular degradation, DNA fragmentation, and blebbing typical of apoptosis. This includes: breakdown of cytosolic and nuclear structural proteins such as actin and lamin; inactivation of the DNArepair enzyme PARP, and activation of the DNA-fragmenting enzyme ICAD; activation of pro-death kinases MEKK and PKC; and activation of additional pro-apoptotic effectors such as Bid (54).

Apoptosis is a tightly, genetically controlled physiological process that typically results in removal of abnormal, damaged, and/or unnecessary cells. It is characterized by compartmentalization of cellular material into membrane-bound “blebs” which are phagocytized by surrounding immune cells (53). This contrasts death by necrosis, which produces a much larger immune response in order to clean up cellular debris (53). Apoptosis is typically studied from induction to completion, implying a requirement for cell elimination. However, it is important to note that incomplete apoptosis can occur from apoptotic signalling mechanisms not intense enough to result in total cell death. Two main signalling pathways regulate the apoptotic process: the death receptor (extrinsic) and the mitochondrial mediated (intrinsic) pathways (53). The extrinsic pathway involves activation of a death-receptor from the tumor necrosis factor (TNF) receptor super-family through their respective ligand (TNF-α, Fas-L, TRAIL) (55). This stimulates assembly of protein scaffolds such as the death-inducing signalling complex (DISC) through interaction of regulatory molecules including TRADD/FADD and procaspase-8 (55). This results in caspase-8 activation leading to cleavage-activation of procaspase-3, and ultimately caspase-3 activation (56). The intrinsic pathway is regulated through the mitochondria and can be induced by toxic stimulants, growth-factor exhaustion, or reactive oxygen species (ROS) (53). These stimuli disrupt electron transport and ATP production, alter mitochondrial membrane polarization, and cause release of proteins such as apoptosis-inducing factor (AIF) and cytochrome c (57). In the cytosol, cytochrome c joins with apoptotic protease activating factor (Apaf-1) and procaspase-9, forming a molecular structure known as the apoptosome (51).

The apoptosome cleavage-activates caspase-9, which in turn activates effector caspases (51). This process is endogenously inhibited by a family of cytosolic proteins known as inhibitors of apoptosis (IAPs), which act on caspases-9 and -3 (58). However, another mitochondrial protein, the second mitochondrial activator of caspases (Smac), is also released into the cytosol which can lead to caspase-3 mediated apoptosis by blocking x-linked-IAP (XIAP) (59). AIF, once released, translocates to the nucleus and results in DNA fragmentation independent of caspase activation (60). A number of accessory proteins are involved in the signalling process. The Bcl-2 protein family (which share common BH3 domain(s)) consists of both activators (Bax, Bak, PUMA) and inhibitors (Bcl-2, Bcl-$_{XL}$) of apoptosis, and function to regulate the release of pro-apoptotic factors such as cytochrome c, AIF, and Smac from the mitochondria (60, 61). Bax translocation from the cytosol to the mitochondria, for example, is considered a typical apoptosis-inducing event which results in depolarization of the mitochondrial membrane and release of caspase-activating molecules (57). Another Bcl-2 protein, Bid, links the extrinsic pathway to the mitochondria, as its cleavage by activated caspase-8 induces the release of cytochrome c (62). Bid can also be cleaved by caspase-2, although its affinity is much lower than that of caspase-8 (63, 64). The classification of caspase-2 as initiator vs. effector has been debated, as it shares substrate specificity with caspases-3 and -7 but is activated through a dimerization mechanism catalyzed by a large multi-protein complex similar to caspases-8 and -9 (65-69). The caspase-2 activating platform, known as the PIDDosome, consists of the proteins PIDD and RAIDD, which bind to each other via their death domains and recruit caspase-2 (68, 70). Additionally, it has been suggested that caspase-2 can directly activate caspase-3 through binding of their pro-domains (71). A final protein expressed at high levels in muscle is apoptosis repressor with caspase recruitment domain (ARC), unique for its ability to interact with both death-receptor and mitochondrial mechanisms (72). ARC exerts its anti-apoptotic effects on death-receptor signalling through inhibition of DISC assembly by directly binding to death domains of adaptor molecules (such as FADD) (73). At the level of the mitochondria, ARC can similarly bind several pro-apoptotic BH3-containing proteins (PUMA, Bax, Bad), thus preventing mitochondrial outer membrane permeablization (MOMP) and subsequent release of cytochrome c, Smac, and other pro-apoptotic factors (73-76). Another important regulator of apoptosis is p53, popularly known as a powerful tumor suppressor. Many cell-death inducing signals converge on p53, which promotes apoptosis through direct protein-protein interactions and by acting as a transcription factor. This multi-functional protein can upregulate transcription of several pro-apoptotic factors such as PUMA, Bax, and PIDD, bind to Bcl-2 at the mitochondria, stimulate ROS production, and shuttle Fas receptor to the cell surface (77-85). Finally, sufficient stress to the endoplasmic reticulum (ER) can lead to Ca^{2+}-induced apoptosis. Here, accumulation of damaged proteins in the ER results in a cellular stress response, leading to Ca^{2+} release and activation of caspase-12 and a class of Ca^{2+}-induced proteases known as calpains (86, 87).

Caspases Link Apoptosis and Differentiation

Despite a definitive role for caspases in cell death, evidence suggests that these proteases may also regulate cellular differentiation. For example, inhibition of caspase-3 activity limits DNA fragmentation and nucleus removal in lens epithelial cells, keratinocytes, and erythrocytes, preventing differentiation in these cell types (88-91). These studies have led researchers to adopt the hypothesis that cellular differentiation may be an abbreviated form of cell death (92). Skeletal muscle differentiation shares many apoptotic similarities and is critically dependent on the activity of caspase-3. In this seminal paper, it was observed that in cultured myoblasts taken from *caspase-3* null mice, as well as in response to chemical inhibition of caspase-3 activity, myotube formation and differentiation were inhibited (50, 93). This finding has since been confirmed by a number of researchers, and is now considered as characteristic of skeletal muscle differentiation as myosin expression (92). The initiator caspases-8, -9 and -12 have also been implicated in skeletal muscle differentiation, and, importantly, these effects are always attributed to their effects on caspase-3. Chemical inhibition of caspase-8 as well as forced expression of dominant-negative FADD greatly reduced myosin and MyoD expression associated with differentiation (94). It was observed that forced reduction in caspase-9 levels prevented transient increases in caspase-3 activity and subsequent differentiation measured by cell fusion events in cultured myoblasts (95). Furthermore, overexpression of Bcl-$_{XL}$ had a similar effect, indicating that typical mitochondrial-mediated apoptotic mechanisms may be responsible for inducing caspase activation during differentiation (95). Likewise, overexpression of ARC in cardiac muscle cells inhibited caspase-3 activity and differentiation (96). Finally, caspase-12 activity associated with endoplasmic reticulum stress has been shown to result in caspase-3 activation and an increase in myotube development (97, 98). Although unrelated to skeletal muscle, several studies have attributed a role for caspase-2 during cell cycle obstruction. It was observed that caspase-2 deficient fibroblasts proliferate at a higher rate and that irradiation-induced growth arrest was partially reduced (99, 100). While the mechanism of caspase activation during skeletal muscle differentiation has not been definitively determined, typical caspase-activating signals such as mitochondrial release of cytochrome c and activation of PUMA have been implicated (101, 102). However, these phenomena are not observed by all researchers (50, 95).

The choice between differentiation and apoptosis in response to caspase activation may be due to the timing, intensity, and location of enzyme activity. In cell types that implicate the mitochondrial apoptotic pathway in differentiation, cytochrome c release occurs slowly, eventually resulting in caspase-3 activation (103, 104). Furthermore, the manner of caspase activation during differentiation has repeatedly been shown to happen transiently (50, 95, 103, 105, 106). In skeletal muscle *in vitro*, a spike in caspase activity is normally observed 1-2 days following the induction of differentiation, with activity returning to the levels observed in myoblasts by days 3-4 (50). These observations are in stark contrast to the pattern of caspase activity typical during apoptosis, which occurs more rapidly and intensely (61, 107). In addition, the quantity of stimuli also likely plays a role in determining whether an apoptotic or differentiation response follows. Treatment of cells with staurosporine (a common inducer of mitochondrial-mediated apoptotic cell death) resulted in the controlled release of cytochrome c that did not lead to apoptosis, indicating that the level of caspase activity required to induce differentiation may be lower than to induce apoptosis (104). These researchers point out that this is a particularly important issue in that complete mitochondrial depletion of cytochrome c would result in an inability to generate ATP, a signal that itself could stimulate cell death-promoting apoptotic signalling (92).

Mechanistic Overlap of Caspases During Skeletal Muscle Differentiation

A possible mechanism that could explain how caspase activation results in differentiation and apoptosis is substrate specificity. In this way, a population of substrates would, when cleaved, result in a "death" response, and a separate set of substrates would produce a "differentiation" response (92). The large number of caspase substrates (>300, (54)) suggest that differentiation- and death-specific pools may exist, although such a comprehensive examination has yet to be performed. Interestingly, typical phenotypic effects of caspase activity during apoptosis such as DNA fragmentation and cleavage of the DNA-repair enzyme PARP have been observed during skeletal muscle differentiation, although the importance of these two events has not been determined (50, 93, 101). Nevertheless, existing data indicate that caspases may target an overlapping substrate population when inducing differentiation or apoptotic signals, in addition to targeting fate-specific substrates (92).

An example of parallel signalling is the caspase activation of protein kinases. Caspase-3 can activate several protein kinases, normally through cleavage of their C-terminal regulatory domain (108, 109). In the study demonstrating decreased muscle differentiation in *caspase-3* null myoblasts, caspase-3 was shown to cleavage-activate mammalian sterile 20-like kinase 1 (MST1) in wild-type cells, and replacement of the activated protein in null myoblasts restored the differentiation program (50). However, MST1 has also been shown to promote apoptosis in response to caspase activation (50, 110). Non-kinase caspase substrates which appear to have "differentiation only" effects have also been reported. The bHLH protein Twist, which can prevent myogenic transcriptional activity of MyoD, has been identified as a caspase-3 substrate, and its cleavage leads to loss of function followed by proteasome-mediated degradation (35, 111, 112). Twist expression is associated with blockade of differentiation and apoptosis in mesodermal cell lines, suggesting that caspase cleavage-inactivation of this protein is prerequisite for execution of either program (111). Caspase activation of these targets provides some indication of the similarity between the differentiation and death signals, yet these proteins represent only a small number of the caspase substrates identified to date.

The mechanisms through which the degree of apoptotic signalling activation is controlled is currently unknown. Interestingly, p53 also promotes skeletal muscle differentiation as indicated by increased mRNA, protein, and transcriptional activity levels during differentiation (40, 113, 114). Replacement of wild-type with dominant-negative p53 inhibited differentiation independent of cell cycle withdrawal and this was due to lack of transcriptional activity (114). In these cells, although pRb is hypophosphorylated (stopping proliferation), levels of pRb are not upregulated, preventing its association with MyoD and resultant activation of the muscle-specific MEF promoters (115). Recently, an interaction between caspase-2 and p53 was reported, a phenomenon resulting in indirect p53 stabilization (116). Using various cell lines with differing p53 expression patterns, these researchers describe a model where caspase-2 cleaves Mdm2, a protein responsible for p53 degradation, resulting in a positive feedback loop of increased p53 activity and hence production of PIDD.

Together, these experiments lead to an appealing conclusion that both the death-receptor and mitochondrial-mediated apoptotic pathways are involved with normal skeletal muscle differentiation, and that caspase activation during this process is a mechanism through which differentiation is actively promoted.

Importance of Mitochondrial Dynamics

A further possible mechanism relating caspase activation and muscle differentiation is through the regulation of mitochondrial morphology. Mitochondria are typically viewed as static, oval-shaped organelles, due to their normal depiction in electron micrographs and textbook-style cartoons. However, mitochondria are highly dynamic, continually undergoing division (fission) and fusion within cells (117). These processes have many important physiological functions, and are necessary for maintenance of mitochondrial homeostasis and normal cellular functioning (118). Regulation of mitochondrial fission and fusion is critically dependent on a relatively small number of genes, the products of which are all large GTPases (118). Fusion relies on the activity of mitofusins 1 and 2 (Mfn1, 2), which are bound to and responsible for fusion of outer mitochondrial membranes, and optic atrophy protein-1 (OPA1), which is bound to and responsible for fusion of inner mitochondrial membranes (118). Fission, meanwhile, seems to depend on the function of a single gene, dynamin-related protein 1 (Drp1), a mainly cytosolic protein which binds to and wraps around mitochondria (by oligomerizing) at fission locations (118).

Alterations to mitochondrial morphology are beneficial to cell health in many ways. An obvious role for mitochondrial fission and fusion is mitochondrial transport (118, 119). Since microtubule transport is much quicker than generating new mitochondria, this allows for on-demand mitochondrial recruitment, as well as redistribution during cellular development (118). Mitochondria must also be appropriately separated in preparation for proliferation, as they are necessary organelles that must be inherited during cell division. Correspondingly, Drp1 activation and production of fragmented (daughter) mitochondria are observed prior to cytokinesis in several cell types (119, 120). Fission is also crucial to the process of mitochondrial biogenesis. Here, large networks divide into smaller, fully functional mitochondria, which can then expand and grow into individual networks, resulting in increased mitochondrial mass (118). These principles may be of great importance in the development of skeletal muscle as not only does cell morphology change drastically, but large increases in mitochondrial content occurs during the transition from single-nucleated myoblast to contracting myotube (121).

Mitochondrial fusion allows for the transfer/sharing of vital regulatory molecules, including membrane proteins, enzymes, and/or matrix components (122, 123). Perhaps most importantly, this includes mitochondrial DNA (mtDNA). In several intriguing experiments both *in vitro* and *in vivo*, researchers demonstrated that healthy mitochondria can transfer mtDNA to cells containing mutated mtDNA, resulting in the presence of mitochondria with normal mtDNA and functional gene products (124-126). Furthermore, if a section of a mitochondrial network is functioning at a lesser capacity, or is at increased risk for damage, fission can selectively target the "bad" portion for degradation by autophagy (127). Interestingly, these researchers observed that, after fission events, fragmented mitochondria were preferentially chosen for degradation when they possessed lower membrane potential (indicating permeablization), and lower levels of OPA1 (indicating less ability to fuse back into the mitochondrial network). As a result, fission and fusion can serve as quality control for mitochondrial health and stability.

Mitochondrial Fission and Apoptosis

Notably, fission is also associated with being pro-apoptotic, typically occurring just prior to caspase activation (128, 129). In response to appropriate stressors, Drp1 translocates to and fragments mitochondria, implicating fission as part of the apoptotic phenotype (129-131). However, fission has also been observed to actively promote the release of pro-apoptotic factors from the mitochondria, such as cytochrome c, primarily through its association with Bax (131-134). Bax is commonly observed to colocalize with both Drp1 and Mfn2 at fission sites, and this is thought to contribute mechanistically to Drp1 function (129). Several experiments show that Drp1 participates in the development of MOMP, as the drop in membrane potential and subsequent release of cytochrome c are diminished in cells with mutant or inhibited Drp1 (130, 134, 135). Importantly, these results indicate that not only is fission associated with apoptosis, but it is a necessary step in the apoptotic process. Drp1 translocation is primarily controlled through post-translational modifications such as phosphorylation and sumoylation. Dephosphorylation induced by Ca^{2+}-stress activation of calcineurin (136, 137), as well as by staurosporine-induced inhibition of PKA (137), has been shown to promote fission by increasing mitochondrial translocation of Drp1. Similarly, mitochondrial-associated Drp1 was observed to by sumoylated in a Bax/Bak dependent manner (131). Accumulation of Drp1 in the mitochondria is also dependent on the pro-apoptotic Bcl-2 family member PUMA for proper translocation (138), implicating p53 and typical apoptotic effectors in the control of mitochondrial fission as well. Interestingly, amplified ARC expression resulted in inhibition of Drp1 mitochondrial accumulation and resulting fission; effects dependent on ARC's interaction with PUMA (138). In another study, mitochondrial release of Smac associated with fission was also inhibited by over-expressing ARC in cardiomyocytes (76).

Given the pro-apoptotic association of mitochondrial fission, upregulation of this process during early differentiation events may be a mechanism of inducing caspase activity. Terminal differentiation of muscle involves generation of large mitochondrial networks (121, 139), and was recently observed to require inhibition of Drp1 (140). However, these researchers did not examine the time course of this occurrence. Although mitochondrial content definitely increases during muscle development to meet the energy demands of its mature form, changes to mitochondrial morphology during the transition from myoblast to myotube may require an elevated level of fission.

Purpose

The contribution of typical apoptotic signalling to skeletal muscle differentiation has not been fully characterized. Furthermore, it is unknown how mitochondrial dynamics influence this process. Due to the drastic changes in cell and mitochondrial morphology that occur during muscle differentiation, it is possible that mitochondrial-mediated apoptotic signalling resulting from transient mitochondrial fission is responsible for activating apoptotic signalling in this context.

Therefore, the purpose of my thesis project was to:

1) Characterize the major apoptotic pathways/molecules during differentiation of skeletal muscle
2) Examine the role caspase-2 during skeletal muscle differentiation, and
3) Determine the importance of mitochondrial fission during skeletal muscle differentiation.

Experiment 1: Apoptotic signalling during skeletal muscle differentiation

An *in vitro* model of skeletal muscle differentiation was used to examine apoptotic signalling during this process. Mouse skeletal myoblasts (C2C12 cells) can be kept in an undifferentiated, proliferative state with appropriate subculturing. Upon incubation in media low in growth factors, these cells spontaneously fuse and differentiate into contracting myotubes. Cells were removed from culture after various lengths of time spent in low growth-factor media, from 6 hours to 15 days, and used for experimental analyses. The degree of differentiation (measured using fluorescent microscopy as well as immunoblotting for MyoD, myosin, and myogenin), cell cycle profile, and several major apoptotic signalling pathways/molecules (AIF, ARC, Bax, Bcl-2, cytochrome c, PUMA, Smac, XIAP, and caspases 2, 3, 8, 9) were assessed during the differentiation process.

Experiment 2: The role of caspase-2 during skeletal muscle differentiation

The effect of caspase-3 inhibition on muscle differentiation is well documented. Preliminary results in our lab show that activation of caspase-2 also occurs during the differentiation process. For this experiment, C2C12 cells were differentiated while being incubated with chemical inhibitors of caspases-2 and -3. For each inhibitor, two concentrations were used: one designed to result in complete enzyme inhibition and one at roughly half this concentration. Cells were collected at various time points as in Experiment #1, and similar immunoblotting, fluorometric enzyme evaluation, cell cycle analysis, and fluorescent microscopy techniques were performed for measuring select markers of differentiation and apoptosis.

Experiment 3: Mitochondrial dynamics during skeletal muscle differentiation

Mitochondrial fission and fusion were assessed by immunoblotting (Drp1 and Mfn2) and fluorescent microscopy in C2C12 cells at various time points of differentiation. The importance of fission during differentiation was tested by incubating cells with two concentrations of mdivi-1, a chemical inhibitor of mitochondrial fission, followed by selective analysis of apoptotic signalling and differentiation as mentioned in Experiment #2.

Hypothesis

It was hypothesized that:

1) Typical pro-apoptotic signalling would be activated early during differentiation, proceeding with an increase in anti-apoptotic signalling. This included:

   a. Early mitochondrial translocation of Bax
   b. Early release of AIF, cytochrome c, and/or Smac
   c. Transient spike in caspase activity
   d. This will be followed by increases in ARC and Bcl-2 as pro-apoptotic signalling diminishes

2) Inhibition of caspase-2 would partially prevent myogenesis due to an inability to activate caspase-3. This would be characterized by:
   a. Decreased/absent transient caspase-3 activity
   b. Decreased markers of differentiation: cell fusion, myosin
3) Chemical inhibition of fission would attenuate apoptotic signalling also resulting in decreased differentiation:
   a. Decreased transient caspase activity
   b. Decreased cell fusion, myosin expression

Methods

Cell Culture

C2C12 mouse skeletal myoblasts (ATCC) were cultured in growth media (GM) consisting of low-glucose Dulbecco's Modified Eagles Medium (DMEM; Hyclone, ThermoScientific) containing 10% fetal bovine serum (FBS; Hyclone, ThermoScientific) with 1% penicillin/streptomycin (Hyclone, ThermoScientific) in 35mm, 100mm, and/or 6-well polystyrene cell culture dishes (BD Biosciences). Cells used were between passages 2-5, and seeded at a density of 650/cm^2. At 1-2 day intervals, culture dishes were aspirated of media, washed with warmed phosphate buffered saline (PBS), and fresh media was replaced. Cells were allowed to proliferate until they reached 70-80% confluence, at which point they were induced to differentiate by replacing GM with differentiation media (DM) consisting of DMEM supplemented with 2% horse serum (Hyclone, ThermoScientific) and 1% penicillin/streptomycin. Cells were isolated and utilized for various biochemical analyses immediately prior to the induction of differentiation (Day 0), and at several following time points (6 hrs, 12 hrs, Day 1, Day 1.5, Day 2, Day 3, Day 5, Day 7, Day 11, Day 15). For cell cycle analyses, subconfluent cells were also collected one day before differentiation was induced (Day -1).

Inhibition of Caspases and Mitochondrial Fission

Chemical inhibition of caspase-2 and caspase-3 activities was performed using the small peptide inhibitors Ac-VDVAD-CHO and Ac-DEVD-CHO, respectively (Enzo Life Sciences) (66, 67). Inhibition of Drp1 activity and subsequent mitochondrial fission was achieved using mdivi-1 (141, 142) (Enzo Life Sciences). All chemicals were diluted in DM prior to their addition to cells, and were added in place of regular DM during differentiation. For these experiments, control cells were given DM with the chemical dilution vehicle dimethyl sulfoxide (DMSO).

Isolation, Fractionation, and Determination of Protein Content

Cells in culture were washed twice with warmed PBS, isolated via trypsinization (0.25% trypsin with 0.2g/L EDTA; ThermoScientific), centrifuged at 1000g for 5 min, resuspended in PBS, and centrifuged once more at 1000g. Whole-cell lysates were generated by adding muscle lysis buffer (MLB; 20mM HEPES, 10mM NaCl, 1.5mM MgCl, 1 mM DTT, 20% glycerol, and 0.1% Triton-X100, pH 7.4) with protease inhibitors (Complete Cocktail; Roche Diagnostics) followed by sonication for 20 seconds.

Additional cells were separated into cytosolic-, mitochondrial-, and nuclear-enriched fractions using differential centrifugation (143-145). Briefly, after trypsinization and washing, cells were incubated in digitonin buffer (PBS with 250mM sucrose, 80mM KCl, and 5mg/mL digitonin) (Sigma-Aldrich) for 5 min on ice. Cells were centrifuged at 1000g for 10 min, the supernatant was collected and centrifuged at 16,000g for 10 minutes to pellet any mitochondrial contamination, and the supernatant from this spin was kept as a pure cytosolic fraction. The remaining pellet (P1) from the 1000g spin was washed in PBS, centrifuged at 1000g for 5 min, resuspended in MLB, and allowed to incubate on ice for 5 min. This was centrifuged at 1000g for 10 min, resulting in a pellet (P2) containing nuclei, and a supernatant (S2) containing mitochondria and other membrane-bound organelles. S2 was centrifuged at 1000g for 10 min to pellet nuclear contamination, with the resulting supernatant kept as the mitochondrial-enriched fraction. The P2 pellet was resuspended in MLB, centrifuged at 1000g for 10 min, resuspended again in MLB, sonicated on ice for 20 seconds, and kept as a nuclear fraction.

Protein content of whole cell lysates and fractions was determined using the BCA protein assay method. Fraction purity was validated by immunoblotting for CuZnSOD (cytosol), MnSOD (mitochondria), and histone H2B (nucleus).

Immunoblotting

As previously performed (143), equal amounts of protein were loaded and separated on 7-12% SDS-PAGE gels, transferred onto PVDF membranes (Bio-Rad Laboratories), and blocked for 1 hr at room temperature or overnight at 4°C with 5% milk-Tris-buffered saline-Tween 20 (milk-TBST). Membranes were then be incubated either overnight at 4°C or for 1 hr at room temperature with primary antibodies against AIF, ARC, ANT, Bcl-2, Bax, cytochrome c, Mfn2, MyoD, p53 (Santa Cruz), CuZnSOD, histone H2B, MnSOD, Smac, XIAP (Enzo Life Sciences), myosin, myogenin, (Developmental Studies Hybridoma Bank), procaspase-3, Drp1 (Cell Signaling), or PUMA (Abcam). Membranes were then washed with TBST, incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (Santa Cruz Biotechnology) for 1 hr at room temperature, washed with TBST, and bands visualized using enhanced chemiluminescence western blotting detection reagents (GE Healthcare) and the ChemiGenius 2 Bio-Imaging System (Syngene). The approximate molecular weight for each protein was estimated using Precision Plus Protein WesternC Standards and Precision Protein Strep-Tactin HRP Conjugate (Bio- Rad Laboratories). Equal loading and quality of transfer was confirmed by staining membranes with Ponceau S (Sigma-Aldrich). Unless otherwise indicated, all immunoblotting was performed and quantified in duplicate.

Fluorometric Caspase Activity Assay

Enzymatic activity of caspase-2, caspase-3, caspase-8, and caspase-9 was determined in cells using the substrates Ac-VDVAD-AMC, Ac-DEVD-AMC, Ac-IETD-AMC, and Ac-LEHD-AMC (Enzo Life Sciences), respectively (143). These fluorogenic substrates are weakly fluorescent but yield highly fluorescent products following proteolytic cleavage by their respective active caspase enzyme. Cells were isolated as mentioned above (using MLB without addition of protease inhibitor cocktail) and incubated in duplicate in black 96-well plates (Costar) with the appropriate fluorogenic substrate at room temperature. Fluorescence was measured using a SPECTRAmax Gemini XS microplate spectrofluorometer (Molecular Devices) with excitation and emission wavelengths of 360 nm and 440 nm, respectively. Caspase activity was normalized to total protein content and expressed as fluorescence intensity in arbitrary units per milligram protein.

Fluorescent Microscopy

Immunofluorescent microscopy was used to visualize nuclei, expression of myosin, and mitochondrial morphology. Cells grown on glass coverslips in culture dishes were removed at appropriate time points and washed 2 x 5min with PBS. Cells were fixed by incubating in 4% formaldehyde-PBS for 10 min, and washed 2 x 5 min with PBS. Next, cells were then permeablized with 0.5% Triton-X100 for 10 min, and washed 2 x 5 min in PBS. Cells were blocked with 10% goat serum (in PBS) for 30 min, incubated with primary antibodies diluted in blocking solution for 1 hr, and washed 2 x 5 min with PBS. Fluorescent-conjugated secondary antibodies (Molecular Probes, Invitrogen Life Technologies) were diluted in blocking solution and incubated with cells for 1 hr, washed 2 x 5 min in PBS, counterstained with DAPI nuclear stain (Molecular Probes) for 5 min, washed 2 x 5 min in PBS, and mounted with Prolong Gold

Antifade Reagent (Molecular Probes). For visualization of mitochondria, live cells were incubated with MitoTracker Green FM (100nM in GM/DM; Molecular Probes) for 30 min at 37°C prior to formaldehyde fixation, and counterstained with DAPI as outlined above. Cells were visualized with an Axio Observer Z1 structured-illumination fluorescent microscope equipped with standard Red/Green/Blue filters, an AxioCam HRm camera, and AxioVision software (Carl Zeiss).

Cell Fusion Index

Using immunofluorescent images stained with myosin and DAPI, the degree of myoblast fusion was determined by counting all nuclei in ten random microscopic fields. The number of nuclei in multi-nucleated cells was divided by the total number of nuclei to give a fusion percentage per field.

Flow Cytometry Analysis of Cell Cycle

Cells were washed with PBS, harvested by trypsinization as described above, centrifuged at 1000g for 5 min, resuspended in PBS, and centrifuged once more at 1000g for 5 min. The supernatant was aspirated, leaving approximately 100µL, and the pellet was resuspended in this volume. While vortexing, 1mL of ice-cold reagent-grade 70% ethanol was slowly added to fix the cells. Following 24 hr fixation, cells were centrifuged at 1000g for 5 minutes. The supernatant was removed and cells were washed twice with PBS. 100µL of RNAase was then added along with 400µL of propidium iodide (PI) solution (50µg/mL in PBS containing 0.1% TritonX) and incubated in the dark at room temperature for 30 minutes. Following this, PI fluorescence was measured using flow cytometry (FACSCalibur, BD Biosciences) and analyzed using Cell Quest Pro software (BD Biosciences).

Cell Counting/Size Analysis

Cell counts were performed to ensure accurate seeding densities as well as assessing the number of apoptotic cells contained in culture media. Using the Z2 Coulter Counter (Beckman-Coulter), cells between 12-19 μm were counted as viable cells and plated at appropriate densities. The number of dead cells was determined by collecting media and PBS washes of cells in culture, and events between 5-12 μm were counted as apoptotic cells.

Statistical Analyses

Unless otherwise stated, all results shown are means ± standard error of the mean (SEM). Data were analyzed using 1-way ANOVA. In experiment #1, post-hoc comparisons were made between day 0 and each subsequent time point using Tukey analysis with $p<0.05$ considered statistically significant. For caspase and mitochondrial fission inhibition experiments, post-hoc comparisons were made between treatment groups within individual time points using Tukey with $p<0.05$ considered statistically significant. Statistical analyses were performed using Microsoft Excel and SPSS.

Results

Characterization of Skeletal Muscle Differentiation

Switching 70-80% confluent C2C12 cells to differentiation media induced spontaneous myoblast fusion and generation of myotubes. Preliminary experiments were performed to determine the appropriate amount of time for proper differentiation to occur *in vitro*. Cells were harvested between 1 and 15 days after addition of differentiation media, and analyzed for terminal differentiation markers through immunoblotting as well as fluorescent microscopy. On day 0, myosin expression and myotube development were undetectable, but both reached their maximum levels after 7 days in differentiation media (Appendix Figure 1). For subsequent experiments, the day 5 and/or 7 time point was considered fully differentiated. The induction of differentiation was associated with an immediate and consistent decrease in the expression level of MyoD protein, with an 87% reduction by day 7 ($p<0.05$, Figure 2A & 2B). Conversely, myogenin protein expression was quickly induced, as its levels on day 2 were 18-fold higher than day 0 ($p<0.05$, Figure 2A & 2B). However, this increase in expression was transient, as myogenin levels decreased by 78% between days 2 and 7, reaching a level similar to day 0 ($p>0.05$, Figure 2A & 2B). Myosin expression, measured to indicate the extent of differentiation, was undetectable on day 0 and increased exponentially until day 7 (Figure 2A). Cell cycle progression as measured using flow cytometry detection of PI fluorescence indicated almost complete growth arrest by day 1 of differentiation (Figure 2C & 2D). The day prior to the induction of differentiation, 56.2% of cells were in G_o/G1 phase, indicating they were not currently proliferating, and the remaining 43.8% of cells were in S and/or M/G2 (Figure 2C & 2D). While a moderate level of cell cycle withdrawal was observed as cells became more confluent, 30.5% of cells were still in a proliferative phase (S and/or M/G2) on day 0 (Figure 2C

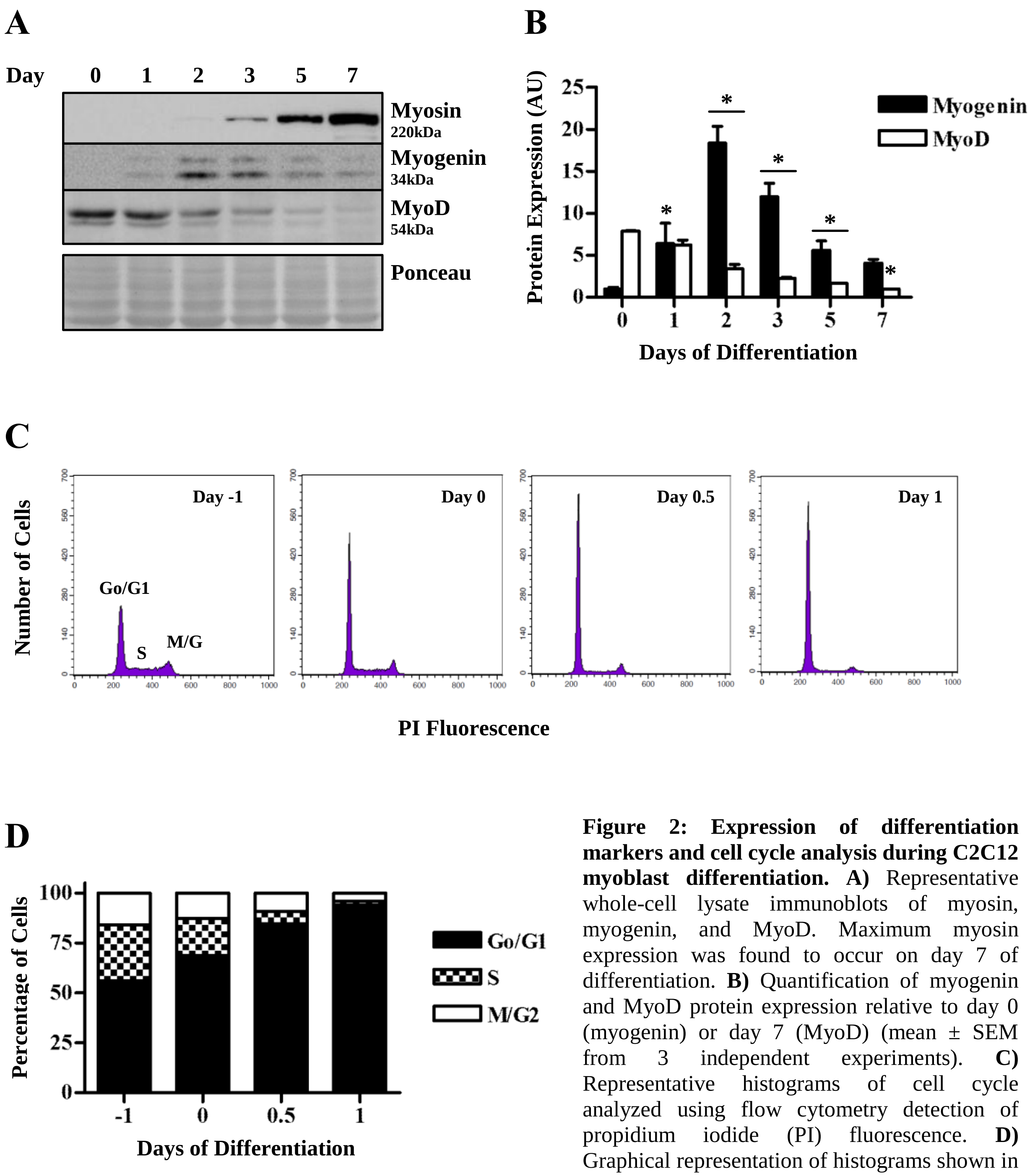
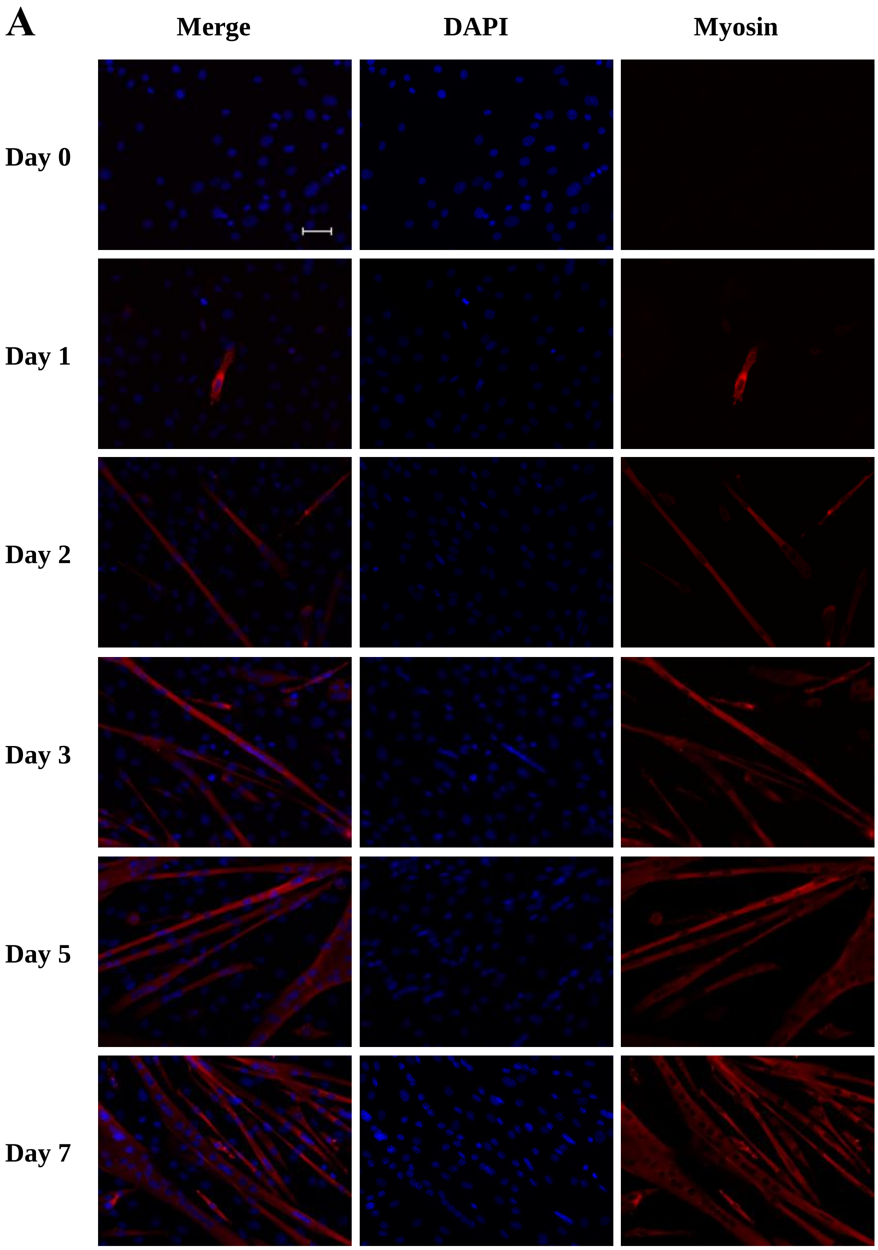


Figure 2: Expression of differentiation markers and cell cycle analysis during C2C12 myoblast differentiation. A) Representative whole-cell lysate immunoblots of myosin, myogenin, and MyoD. Maximum myosin expression was found to occur on day 7 of differentiation. **B)** Quantification of myogenin and MyoD protein expression relative to day 0 (myogenin) or day 7 (MyoD) (mean ± SEM from 3 independent experiments). **C)** Representative histograms of cell cycle analyzed using flow cytometry detection of propidium iodide (PI) fluorescence. **D)** Graphical representation of histograms shown in (C), highlighting the growth arrest of C2C12 cells by day 1 of differentiation. *p<0.05 compared to day 0.



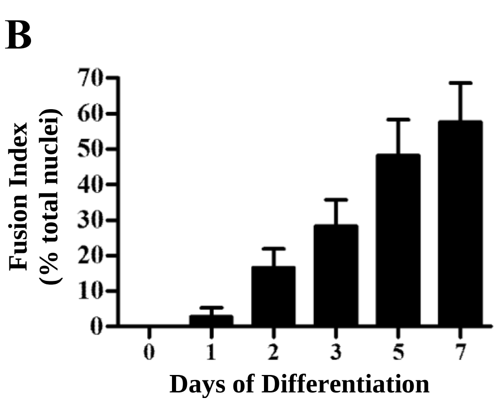


Figure 3: Cell morphology during C2C12 myoblast differentiation. A) Fluorescent microscopy was used to visualize cells at various time points of differentiation. DAPI stains nuclei blue, while myosin expression is shown in red. Bar represents 50µm. **B)** Cell fusion index was calculated by dividing the number of nuclei contained in multi-nucleated cells by the total number of nuclei in ten random microscopic fields (mean ± SD).

& 2D). However, cell cycle was shown to be arrested in almost all cells by da y 1 as 94% existed in G_o/G1 leaving only 6% in an actively dividing phase (Figure 2C & 2D). Measurement of cell cycle was discontinued at this time point as flow cytometry analyses are complicated by multi-nucleated cells such as myotubes. Cell morphology visualized using immunofluorescent microscopy detection of myosin showed dramatic myotube development during differentiation which appeared to peak on day 7 (Figure 3 & Append ix Figure 1). Assessment of cell fusion events by measuring the percentage of nuclei contained in multi-nucleated cells also demonstrated a progressive increase until day 7 (Figure 3B). On this day, 56.7% of nuclei were located in multi-nucleated cells.

Caspase Activity

As discussed above, caspases have been observed to be temporarily activated during skeletal muscle differentiation (92). The activities of caspases-8 and -9 did not change during differentiation ($p>0.05$, Figure 4). However, caspase-2 activity began increasing 12 hrs after inducing differentiation, and remained increased by 45-51% until day 1.5 ($p<0.05$, Figure 4). Likewise, a progressive elevation in caspase-3 activity was observed, reaching a 2.5-fold increase above day 0 levels by day 1.5 ($p<0.05$). For both caspase-2 and -3, activity returned to the levels observed on day 0 once cells became fully differentiated (Figure 4).

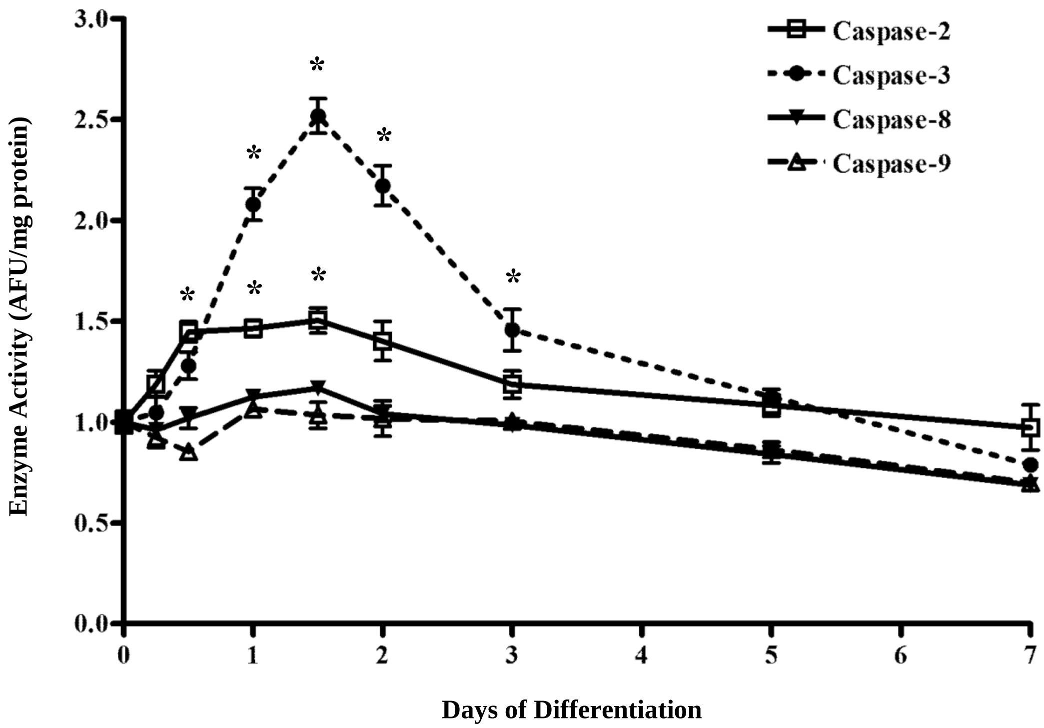


Figure 4: Caspase activity during C2C12 myoblast differentiation. Caspase activity was measured using specific fluorogenic substrates. Activity for each enzyme has been expressed relative to protein content and normalized to levels observed on day 0. *$p<0.05$ compared to day 0 (mean ± SEM from 3 independent experiments).

Pro-Apoptotic Signalling

The mechanism of this characteristic transient spike in caspase activity has not yet been determined, with some researchers observing increases in typical upstream apoptotic signalling while others have not. To further elucidate potential causes, the expression level and subcellular localization of several pro-apoptotic signalling proteins was measured during differentiation. In whole-cell lysates, the expression levels of Bax and PUMA were increased by 16% and 23% respectively, on day 1 compared to day 0 ($p<0.05$, Figure 5A & 5B). After this initial increase, Bax levels stabilized near those observed on day 0, with moderate, statistically insignificant fluctuations observed on subsequent days (<8%, $p>0.05$). Levels of procaspase-3 similarly

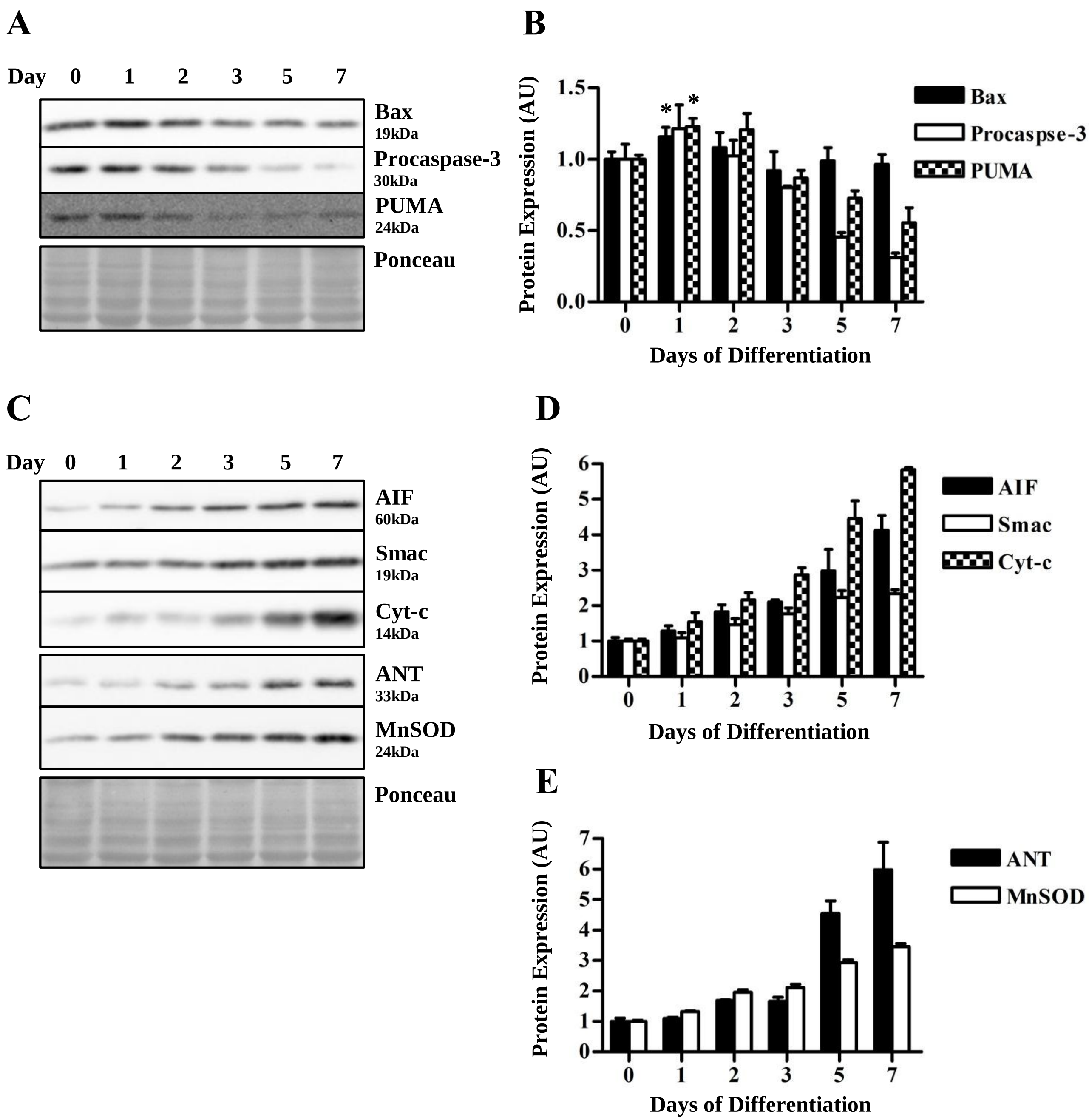


Figure 5: Pro-apoptotic protein expression during C2C12 myoblast differentiation. A) Representative immunoblots of whole-cell protein expression of Bax, procaspase-3, and PUMA. **B)** Quantification of Bax, procaspase-3, and PUMA protein expression levels expressed relative to day 0. **C)** Representative immunoblots of whole-cell protein expression of AIF, Smac, cytochrome c (Cyt-c), ANT, and MnSOD. **D)** Quantification of mitochondrial-located apoptotic protein expression levels expressed relative to day 0. Although expression of these proteins increased dramatically during differentiation, this is likely due to increased mitochondrial content as demonstrated by parallel increases in ANT and MnSOD shown graphically in **(E)**. *$p<0.05$ compared to day 0. (mean ± SEM from 3 independent experiments).

increased slightly on day 1 by 21%, although this change did not achieve statistical significance (Figure 5A & 5B). Unlike Bax, whose expression seemed to stabilize as differentiation continued, both PUMA and procaspase-3 decreased progressively after their day 1 peak by 54% and 74% by day 7, respectively (Figure 5A & 5B). Whole-cell protein expression levels of the mitochondrial-located factors AIF, Smac, and cytochrome c (Cyt-c) gradually increased during differentiation (Figure 5C & 5D). From day 0 to day 7, this resulted in a 4.1-fold increase in AIF, a 2.3-fold increase in Smac, and a 5.8-fold increase in cytochrome c (Figure 5C & 5D). While these changes are very dramatic, they are most likely due to an increase in total mitochondrial content, as expression of the mitochondrial proteins ANT and MnSOD were observed to increase in a similar manner (Figure 5C & 5E).

Anti-Apoptotic Signalling

Expression of the anti-apoptotic proteins XIAP, ARC, and Bcl-2 were analyzed in whole-cell lysates at various time points during differentiation. Levels of XIAP decreased progressively from day 0, with an 86% drop observed by day 7 ($p<0.05$, Figure 6A & 6B). Conversely, ARC expression rose progressively during differentiation, undergoing a 5.6-fold increase between days 0 and 7 ($p<0.05$, Figure 6A & 6B). Whole-cell Bcl-2 levels also increased during differentiation, however, a peak was observed on day 2, when expression was 2.5-fold higher compared to day 0 ($p<0.05$, Figure 6A & 6B).

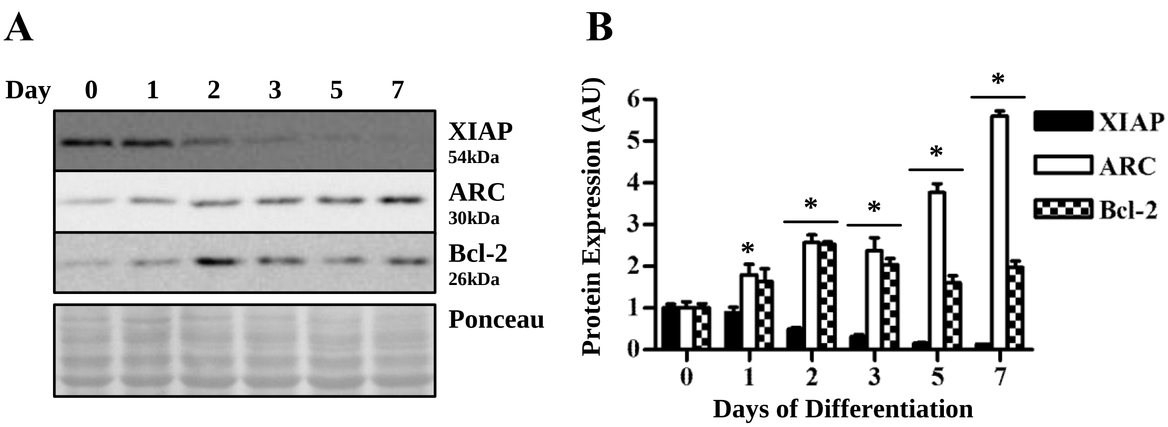


Figure 6: Anti-apoptotic protein expression during C2C12 myoblast differentiation. A) Representative immunoblots of whole-cell protein levels of XIAP, ARC, and Bcl-2. **B)** Quantification of protein expression levels relative to day 0. *p<0.05 compared to day 0. (mean ± SEM from 3 independent experiments).

Cellular Translocation of Apoptotic Factors

As the apoptosis-inducing role of many of these proteins depends on their cellular location, additional C2C12 cells were separated into subcellular fractions at various time points during the differentiation process. Mitochondrial translocation of Bax, mentioned above to precede membrane permeablization, was progressively increased during differentiation (Figure 7A & 7B). Importantly, a 2.6-fold increase was observed on day 1.5 ($p<0.05$, Figure 7A). However, mitochondrial levels of Bcl-2, a protein which opposes the pro-apoptotic functions of Bax, similarly increased 2.8-fold by day 1.5 ($p<0.05$, Figure 7A & 7B). As a result, the mitochondrial Bax:Bcl-2 ratio, considered a marker of apoptotic susceptibility, did not significantly change during early differentiation events (< 2 days, before the spike in caspase activity ($p>0.05$, Figure 7C). However, similar to analyses performed in whole-cell lysates, mitochondrial Bcl-2 levels peaked on day 2 with a 3.5-fold increase above day 0 ($p<0.05$), followed by a decrease as differentiation continued (Figure 7A & 7B). This led to a significantly increased Bax:Bcl-2 ratio on subsequent days of differentiation ($p<0.05$, Figure 7C).

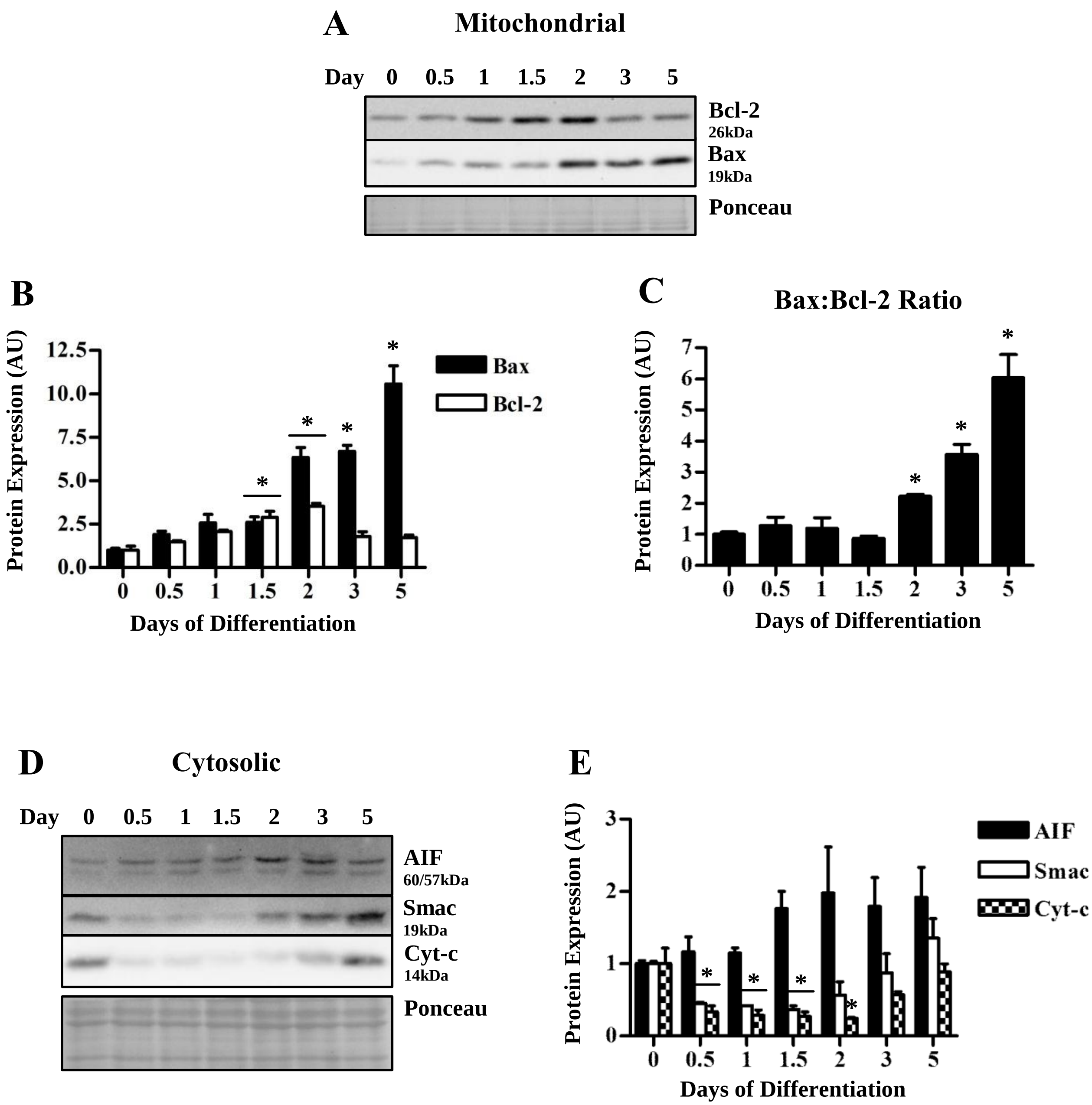


Figure 7: Translocation/subcellular location of apoptotic factors during C2C12 myoblast differentiation. A) Representative immunoblots of Bcl-2 and Bax in mitochondrial-enriched subcellular fractions. **B)** Quantification of mitochondrial Bax and Bcl-2 relative to day 0. **C)** Quantification of the Bax:Bcl-2 ratio relative to day 0. **D)** Representative immunoblots of cytosolic levels of mitochondrial pro-apoptotic factors AIF, Smac, and cytochrome c (Cyt-c). The 57kDa AIF band corresponds to its activated form (see discussion for details). **E)** Quantification of cytosolic protein expression levels relative to day 0. *$p<0.05$ compared to day 0. (mean ± SEM from 3 independent experiments).

The mitochondria's role in promoting apoptosis culminates in its release of several factors into the cytosol. During differentiation, cytosolic levels of AIF rose slightly, although this measurement was highly variable and was not significant (Figure 7C & 7D). Perhaps most notably, cytosolic Smac and cytochrome c significantly decreased 12 hours after induction of differentiation ($p<0.05$, Figure 7C & 7D). This response lasted 36-48 hours as cytosolic Smac levels were reduced 55-63% between day 0.5 and 1.5 ($p<0.05$), while cytosolic cytochrome c levels were reduced 67-75% between day 0.5 and 2 ($p<0.05$) (Figure 7C & 7D). Surprisingly, this response was transient and cytosolic levels of both proteins returned to those observed on day 0 once cells became fully differentiated (Figure 7C & 7D).

Mitochondrial Dynamics

To analyze the molecular control of mitochondrial fission and fusion during differentiation, immunoblotting was performed for mitofusin 2 (Mfn2) and Drp1. Mfn2 levels decreased by 65% in whole-cell lysates during differentiation from day 0 to day 7 ($p<0.05$, Figure 8A & 8B). On the other hand, Drp1 expression was increased 2.4-fold above day 0 levels by day 7 ($p<0.05$, Figure 8A & 8B). Notably, 49% of this change was observed between days 1 and 2 (Figure 8A & 8B). Because Drp1 is mainly cytosolic and must translocate to the mitochondria to induce fission, immunoblotting was also performed on mitochondrial fractions during differentiation. Mitochondrial levels of Drp1 increased by 2.6-fold by day 2 of differentiation ($p<0.05$, Figure 8C & 8D). By day 3, mitochondrial Drp1 levels returned to those observed on day 0 ($p>0.05$, Figure 8C & 8D).

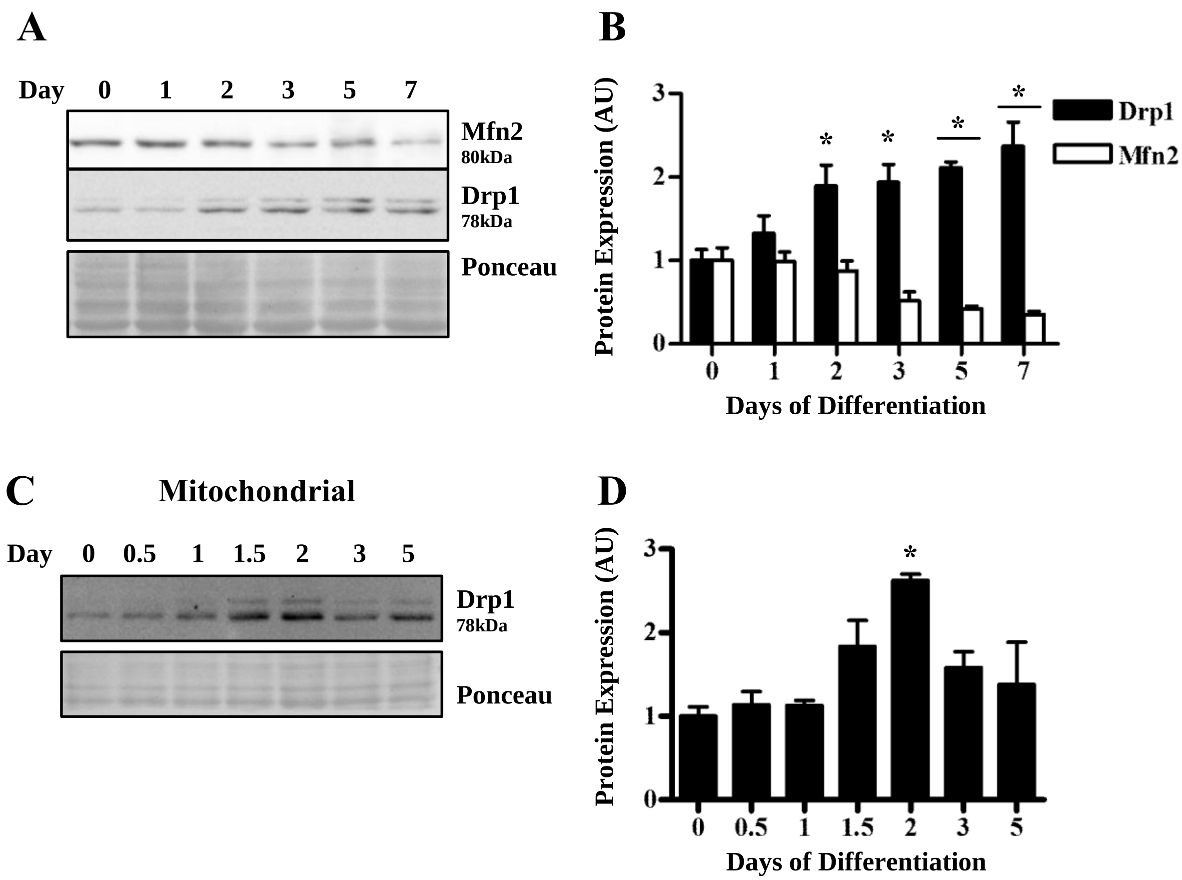


Figure 8: Regulation of mitochondrial dynamics during C2C12 myoblast differentiation. A) Representative immunoblots of whole-cell protein expression of Mfn2 and Drp1. **B)** Quantification of protein expression levels expressed relative to day 0. **C)** Representative immunoblot of Drp1 in mitochondrial-enriched fractions. **D)** Quantification of mitochondrial Drp1 levels. *$p<0.05$ compared to day 0. (mean ± SEM from 3 independent experiments).

Caspase-3 Inhibition Results in Decreased Skeletal Muscle Differentiation

As other researchers have demonstrated a requirement for caspase-3 activity during skeletal muscle differentiation, we first characterized the differentiation of our C2C12 cells in the presence of a chemical inhibitor of caspase-3 activity, Ac-DEVD-CHO. Cells were induced to differentiate upon reaching 70-80% confluence by switching to differentiation media with 30μM Ac-DEVD-CHO (casp-3 inh 30μM), 75μM Ac-DEVD-CHO (casp-3 inh 75μM), or DMSO (control). Control cells underwent significant myotube development between day 0 and day 5, similar to what we previously observed (Figure 9A). However, this process was progressively inhibited in myoblasts incubated with caspase-3 inhibitor (Figure 9A). With respect to cell fusion events, administration of 30μM Ac-DEVD-CHO resulted in a 44% and 12% decrease on days 2 and 5, respectively, compared to control cells (Figure 9B). This effect was more pronounced at 75μM, with a 72% and 54% reduction in cell fusion observed on days 2 and 5, respectively, compared to control cells (Figure 9B).

Both concentrations of caspase-3 inhibitor significantly reduced the activities of caspases-2 and -3 (Figure 10A). Similar to our previous data, caspase-2 activity transiently increased 50% above day 0 levels in control cells during the first 2 days of differentiation (Figure 10A). After 12 hours, caspase-2 levels were 49% and 50% lower in cells given 30μM and 75μM caspase-3 inhibitor, respectively ($p<0.05$, Figure 10A). Caspase-2 levels remained significantly decreased 31-55% in both treatment groups compared to control cells until day 3 ($p<0.05$, Figure 10A). Once cells reached full differentiation (day 5), caspase-2 activity levels in cells receiving caspase-3 inhibitor were no longer different than control ($p>0.05$, Figure 10A). Not surprisingly, caspase-3 activity levels were dramatically decreased in response to Ac-DEVD-CHO treatment. Similar to our previous data, caspase-3 activity transiently increased ~2.2-fold in control cells

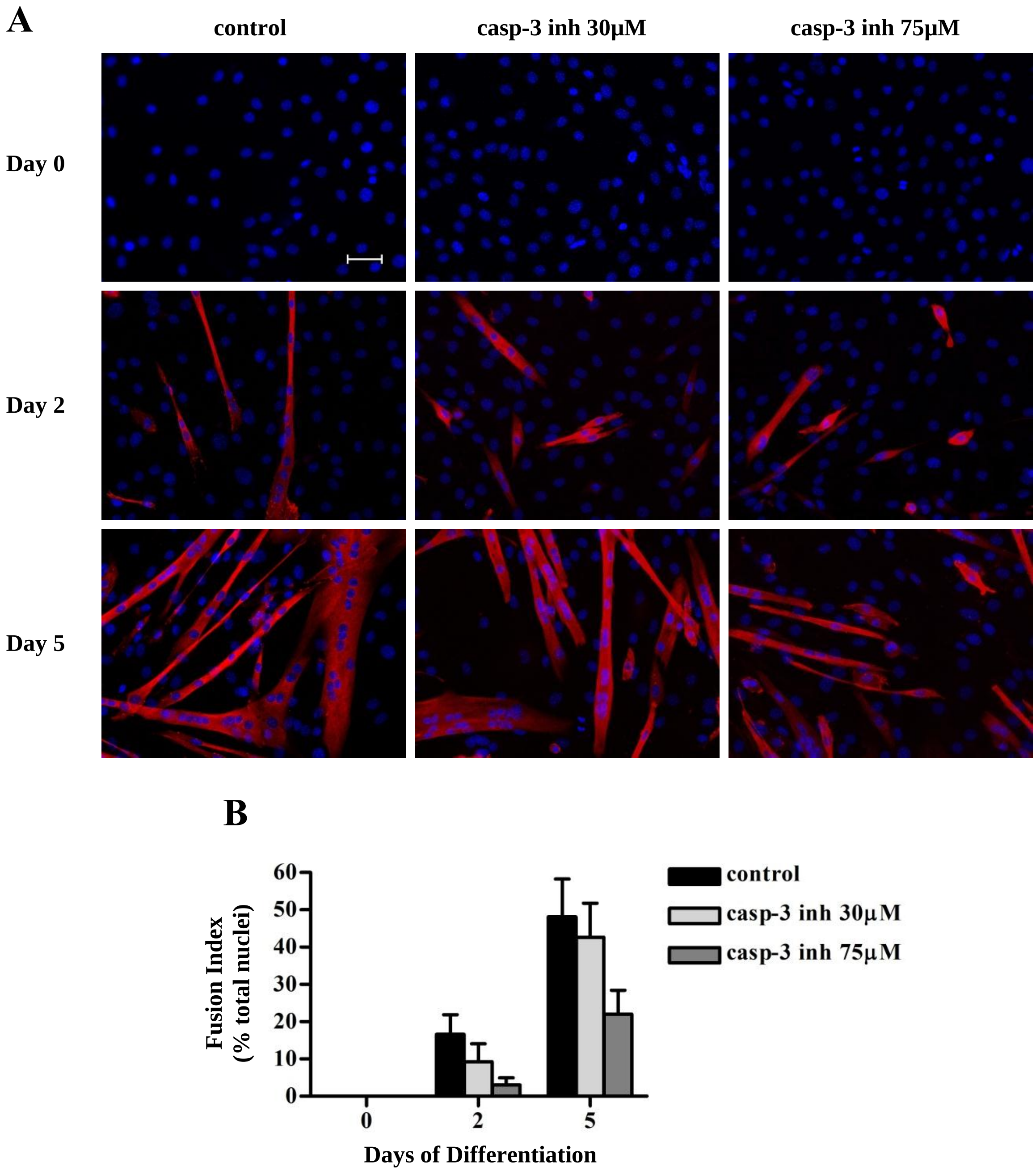


Figure 9: Inhibition of caspase-3 results in decreased myotube development. A) Fluorescent microscopy was used to visualize cells at various time points of differentiation. DAPI stains nuclei blue, while myosin expression is shown in red. Bar represents 50μm. **B)** Cell fusion index was calculated by dividing the number of nuclei contained in multi-nucleated cells by the total number of nuclei in ten random microscopic fields (mean ± SD). Increasing concentrations of caspase-3 inhibitor led to a progressive drop in myotube development and cell fusion.

early during differentiation (Figure 10A). After 24 hours, caspase-3 activity levels were 70% and 71% lower in cells given 30µM and 75µM Ac-DEVD-CHO, respectively, compared to control cells ($p<0.05$, Figure 10A). Caspase-3 activity remained significantly decreased in both treatment groups compared to control until day 3 ($p<0.05$, Figure 10A). By day 5, caspase-3 activity levels returned to those observed on day 0 and were not different between groups ($p>0.05$, Figure 10A).

A dramatic reduction in myogenic markers measured using immunoblotting was also observed in response to caspase-3 inhibition. In control cells, expression of myosin and myogenin changed in similar patterns to what we previously observed (Figure 10B & 10C). Administration of 30µM caspase-3 inhibitor led to 39% and 34% reduction of myosin expression by days 3 and 5, respectively, compared to control cells ($p<0.05$, Figure 10B & 10C). Likewise, myosin levels were 74% and 58% reduced in cells that received 75µM caspase-3 inhibitor compared to control cells on days 3 and 5, respectively ($p<0.05$, Figure 10B & 10C). The induction of myogenin expression was not affected by 30µM caspase-3 inhibition, as levels were not different from control cells on days 1-3 ($p>0.05$, Figure 10B & 10C). However, in these cells myogenin expression did not decrease during terminal differentiation, and were 2.6-fold higher than control cell levels on day 5 ($p<0.05$, Figure 10B & 10C). Conversely, the induction of myogenin was prevented by 75µM caspase-3 inhibitor, as expression levels were 50% and 46% reduced on day 1 and day 2, respectively, compared to control cells ($p<0.05$, Figure 10B & 10C).

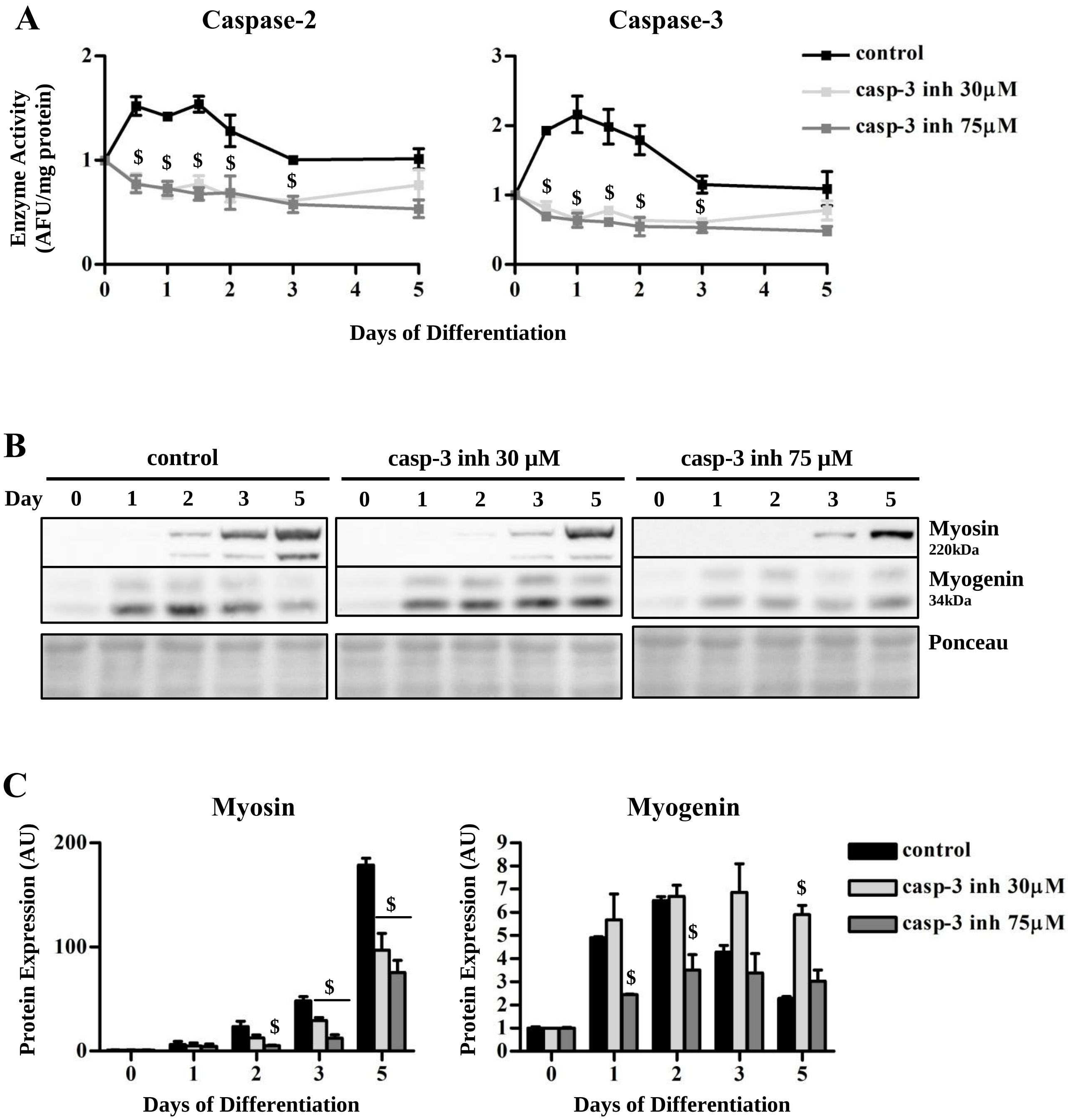


Figure 10: Changes to caspase activity and differentiation markers with caspase-3 inhibition. A) Caspase-2 and -3 activity relative to day 0 levels. **B)** Representative whole-cell lysate immunoblots of myosin and myogenin. **C)** Quantification of myosin and myogenin protein expression. \$p<0.05 from control within a time point. (mean ± SEM from 3 independent experiments)

Caspase-2 Inhibition Results in Decreased Skeletal Muscle Differentiation

The involvement of caspase-2 during skeletal muscle differentiation has not been investigated. To examine this, we pharmacologically inhibited caspase-2 activity with the chemical Ac-VDVAD-CHO during C2C12 differentiation. Cells were induced to differentiate upon reaching 70-80% confluence by switching to differentiation media with 30μM Ac-VDVAD-CHO (casp-2 inh 30μM), 75μM Ac-VDVAD-CHO (casp-2 inh 75μM), or DMSO (control). Similar to our previous experiments, control cells underwent significant myotube development between day 0 and day 5 (Figure 11A). However, this process was progressively inhibited in cells incubated with caspase-2 inhibitor (Figure 11A). Administration of 30μM Ac-VDVAD-CHO resulted in a 38% and 31% decrease in cell fusion on days 2 and 5, respectively, compared to control cells (Figure 11B). This effect was more prominent at 75μM, with a 58% and 48% reduction in cell fusion observed on days 2 and 5, respectively, compared to control cells (Figure 11B).

Both concentrations of Ac-VDVAD-CHO led to reductions in the activities of caspases-2 and -3 early during differentiation (Figure 12A). Similar to our previous data, caspase-2 activity transiently increased ~50% in control cells during the first 2 days of differentiation (Figure 12A). After 24 hours, caspase-2 levels were 25% and 33% lower in cells given 30μM and 75μM caspase-2 inhibitor, respectively ($p<0.05$, Figure 12A). Caspase-2 levels remained significantly decreased 23-36% in both treatment groups compared to control cells until day 1.5 ($p<0.05$, Figure 12A). On days 2-5, caspase-2 activity was not different between groups, as the levels in control cells returned to those observed on day 0 ($p>0.05$, Figure 12A). Similar to previous experiments, caspase-3 activity transiently increased ~2.2-fold in control cells early during differentiation (Figure 12A). After 24 hours, caspase-3 activity levels were 32% and 35% lower

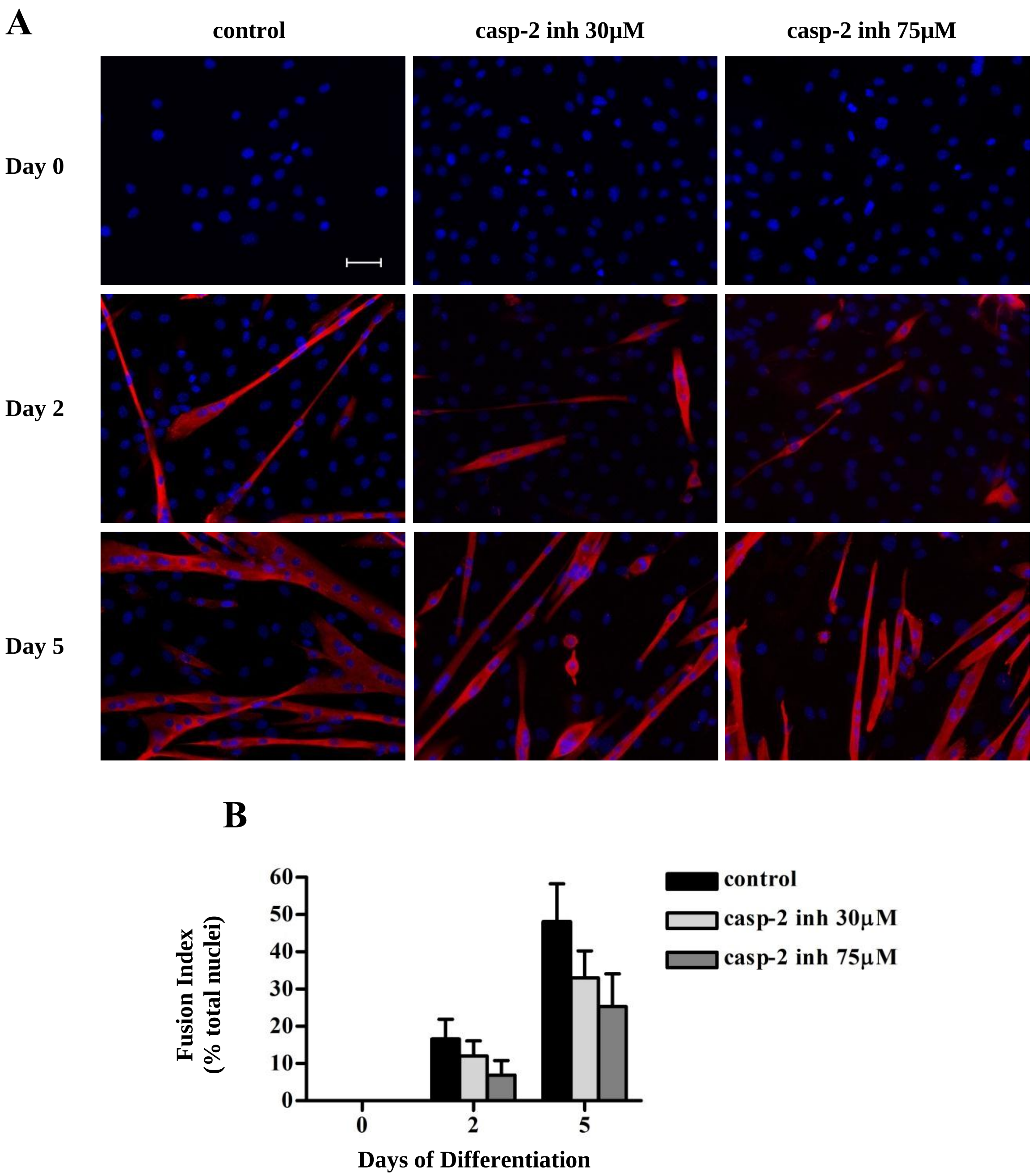


Figure 11: Inhibition of caspase-2 results in decreased myotube development. A) Fluorescent microscopy was used to visualize cells at various time points of differentiation. DAPI stains nuclei blue, while myosin expression is shown in red. Bar represents 50µm. **B)** Cell fusion index was calculated by dividing the number of nuclei contained in multi-nucleated cells by the total number of nuclei in ten random microscopic fields (mean ± SD). Increasing concentrations of caspase-2 inhibitor led to a progressive drop in myotube development and cell fusion.

in cells given 30μM and 75μM Ac-VDVAD-CHO, respectively, compared to control cells ($p<0.05$, Figure 12A). Caspase-3 activity was similarly decreased in both treatment groups on day 2 ($p<0.05$, Figure 12A). On days 3-5, caspase-3 activity levels returned to those observed on day 0 and were not different between groups ($p>0.05$, Figure 12A).

A reduction in myogenic markers measured using immunoblotting was also observed in response to caspase-2 inhibition. In control cells, expression of myosin and myogenin changed similar to what we previously observed (Figure 12B & 12C). Administration of 30μM caspase-2 inhibitor led to a 35% reduction in myosin expression on day 5 compared to control cells ($p<0.05$, Figure 12B & 12C). Likewise, myosin levels were 71% and 70% reduced in cells that received 75μM Ac-VDVAD-CHO compared to control cells on days 3 and 5, respectively ($p<0.05$, Figure 12B & 12C). Myogenin expression was not affected by 30μM caspase-2 inhibition, as levels were not different from control on any day of differentiation ($p>0.05$, Figure 12B & 12B). However, the induction of myogenin was prevented by 75μM caspase-2 inhibitor, as expression levels were 51% and 58% reduced on day 1 and day 2, respectively, compared to control cells ($p<0.05$, Figure 12B & 12C).

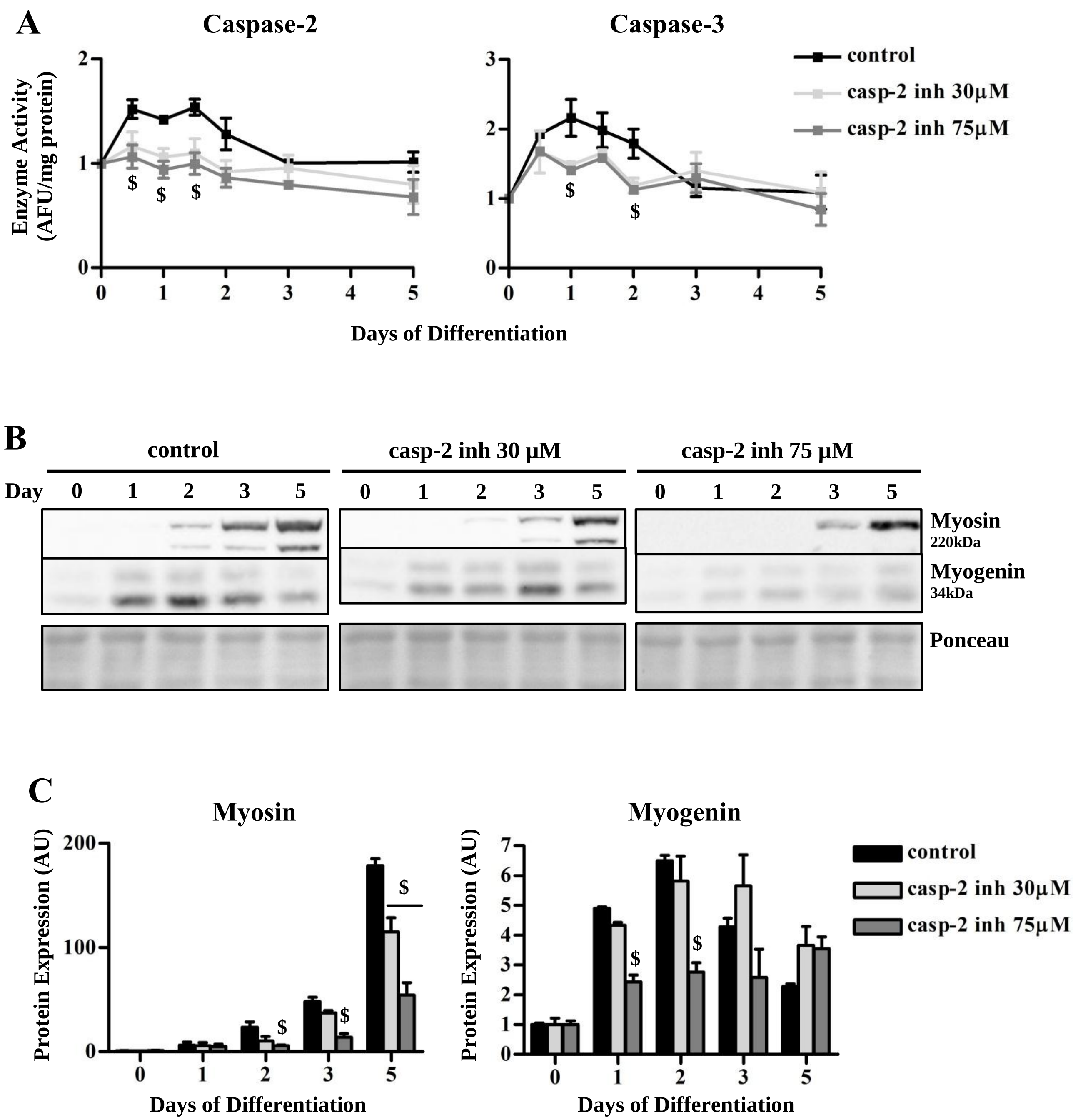


Figure 12: Changes to caspase activity and differentiation markers with caspase-2 inhibition. A) Caspase-2 and -3 activity relative to day 0 levels. **B)** Representative whole-cell lysate immunoblots of myosin and myogenin. **C)** Quantification of myosin and myogenin protein expression. $p<0.05 from control within a time point. (mean ± SEM from 3 independent experiments)

Mitochondrial Fission is Required for Skeletal Muscle Differentiation

To investigate the importance of mitochondrial fission during skeletal muscle differentiation, cells were induced to differentiate in the presence of the chemical inhibitor of Drp1, mdivi-1. As fission is known to promote apoptotic signalling, the efficacy of this chemical was originally tested by examining its ability to inhibit the cellular apoptotic response to treatment with staurosporine (141). In COS cells, the ID_{50} of mdivi-1 was determined to be 50µM (141). We performed similar testing in C2C12 cells by assessing the ability of mdivi-1 to inhibit mitochondrial fission and apoptosis in response to staurosporine (Appendix Figure 2). Treatment with staurosporine alone led to immediate and complete apoptotic cell death (Appendix Figure 2). The addition of mdvi-1 led to a concentration dependent inhibition of mitochondrial fragmentation and cell blebbing (Appendix Figure 2), from which we selected two concentrations to use during differentiation. C2C12 cells were induced to differentiate upon reaching 70-80% confluence by switching to differentiation media with 20µM mdivi-1, 50µM mdivi-1, or DMSO (control). As in previous experiments, control cells underwent significant myotube development between day 0 and day 5 (Figure 13A). However, this process was inhibited in myoblasts given mdivi-1, and was almost absent at a concentration of 50µM (Figure 13A). With respect to cell fusion events, administration of 20µM mdivi-1 resulted in a 50% and 37% decrease on days 2 and 5, respectively, compared to control cells (Figure 13B). This effect was quite severe at 50µM, with a 94% and 93% reduction in cell fusion observed on days 2 and 5, respectively, compared to control cells (Figure 13B).

Both concentrations of mdivi-1 led to dramatic increases in the activities of caspases-2 and -3, although this response was greater in cells given 50µM (Figure 14A). Similar to our previous data, caspase-2 activity transiently increased ~35% in control cells early during

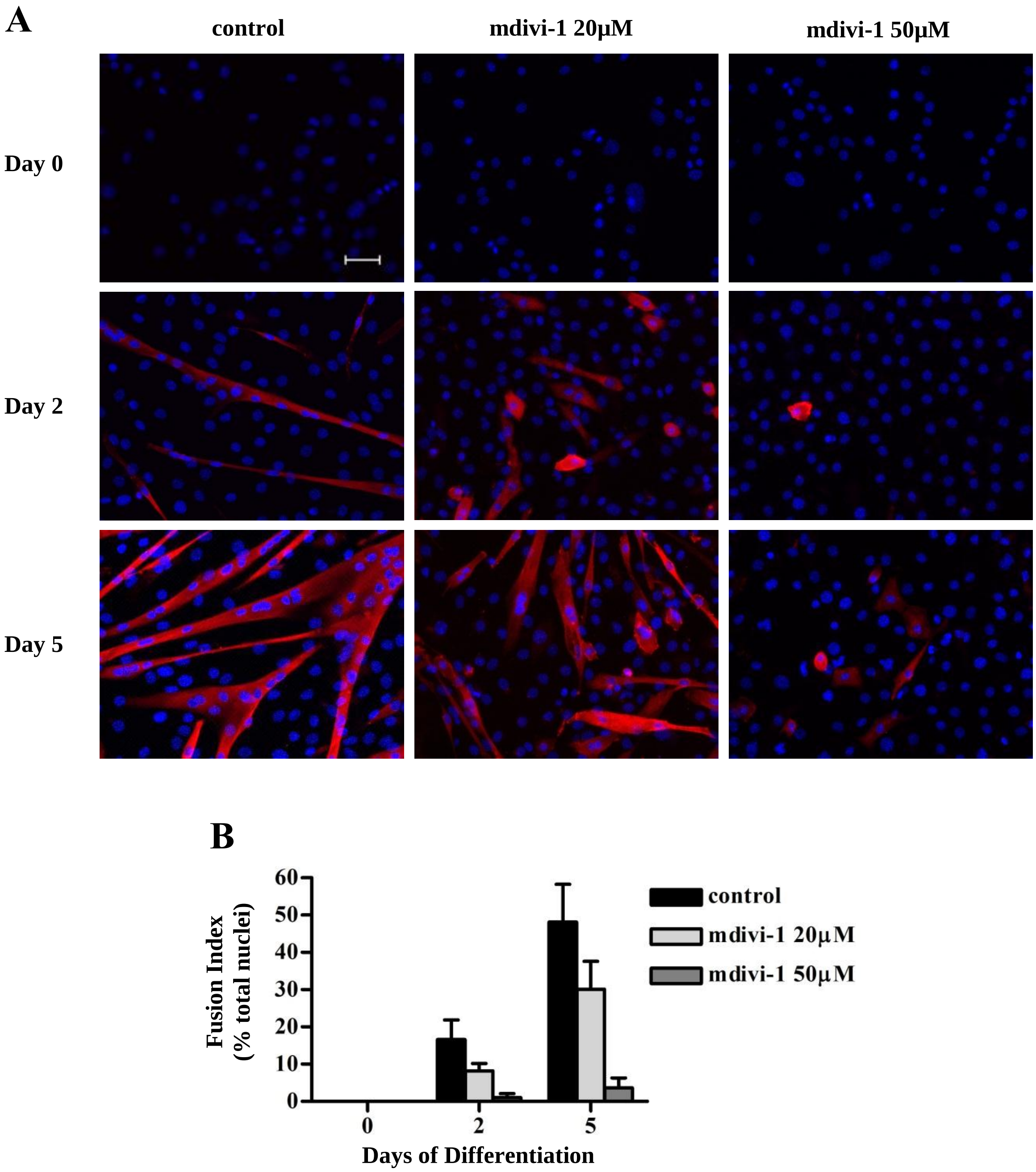


Figure 13: Inhibition of mitochondrial fission results in decreased myotube development. A) Fluorescent microscopy was used to visualize cells at various time points of differentiation. DAPI stains nuclei blue, while myosin expression is shown in red. Bar represents 50μm. **B)** Cell fusion index was calculated by dividing the number of nuclei contained in multi-nucleated cells by the total number of nuclei in ten random microscopic fields (mean ± SD). Increasing concentrations of mdivi-1 led to a progressive drop in myotube development and cell fusion.

differentiation (Figure 14A). After 12 hours, caspase-2 levels were 91% and 93% higher in cells given 20µM and 50µM mdivi-1, respectively ($p<0.05$, Figure 14A). On day 1, activity levels in 20µM cells began to decline, but were still 45% higher than control cells ($p<0.05$, Figure 14A). However, caspase-2 activity continued to increase in cells given 50µM mdivi-1 on day 1, reaching levels 2.4-fold higher than control cells, and 63% higher than 20µM ($p<0.05$, Figure 14A). By day 2, caspase-2 activity levels in all groups returned to those observed on day 0 and were not different between treatments ($p>0.05$, Figure 14A). Similar changes were observed for caspase-3 activity in response to mdivi-1 treatment. Again, caspase-3 activity transiently increased ~2.4-fold in control cells early during differentiation (Figure 14A). After 12 hours, caspase-3 levels were 2.8- and 3.1-fold higher in cells given 20µM and 50µM mdivi-1, respectively, compared to control cells ($p<0.05$, Figure 14A). On day 1, activity levels in 20µM cells began to decline, but were still 65% higher than control cells ($p<0.05$, Figure 14A). However, caspase-3 activity continued to increase in cells given 50µM mdivi-1 on day 1, reaching levels 3.2-fold higher than control cells, and 92% higher than 20µM ($p<0.05$, Figure 14A). By day 3, caspase-3 activity levels returned to those observed on day 0 and were not different between treatment groups ($p>0.05$, Figure 14A).

A dramatic reduction in myogenic markers measured using immunoblotting was also observed in response to mdivi-1 treatment. In control cells, expression of myosin and myogenin changed in similar patterns to what was previously observed (Figure 14B & 14C). Administration of 50μM mdivi-1 almost completely prevented the induction of myosin, and its expression was significantly lower than control cells on days 2-5 ($p<0.05$, Figure 14B & 14C). While myosin was produced in cells given 20μM, its levels on day 5 were significantly lower compared to control cells ($p<0.05$, Figure 14B & 14C). Likewise, 50μM mdivi-1 also prevented the induction of myogenin, as levels did not increase above day 0, and were lower than control cells at every subsequent time point ($p<0.05$, Figure 14B & 14C). Although myogenin expression did increase 3.6-fold by day 3 in cells given 20μM, its levels were 67% and 53% lower than those observed in control cells on days 2 and 3, respectively ($p<0.05$, Figure 14B & 14C).

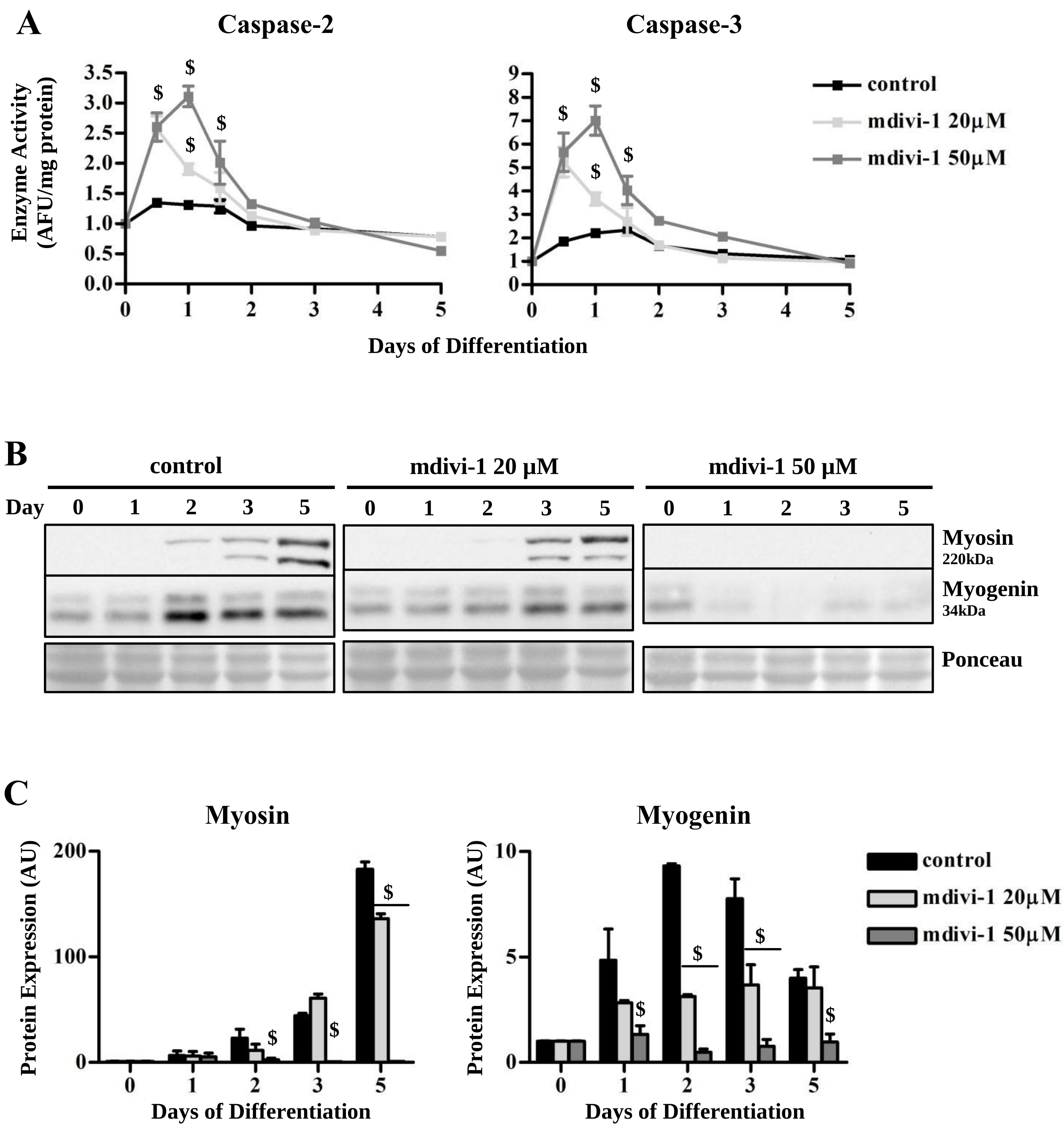


Figure 14: Changes to caspase activity and differentiation markers with inhibition of mitochondrial fission. A) Caspase-2 and -3 activity realtive to day 0 levels. **B)** Representative whole-cell lysate immunoblots of myosin and myogenin. **C)** Quantification of myosin and myogenin protein expression. $p<0.05 from control within a time point. (mean ± SEM from 3 independent experiments).

Discussion

That interplay exists between cellular signals regulating both apoptosis and differentiation is not surprising, nor is it a recently discovered phenomenon. Indeed, the ability of extracellular signals such as hormones to produce either response has been known for some time (146-148). In fact, even typically pro-death ligands such as TNF-α have long been implicated in promoting cellular functions other than apoptosis (149). Often, these overlapping effects are due to variations in receptor activation, resulting in differential responses from complex, multi-functional cellular signalling families such as NF-κB, p38/MAPK, and/or JNK (150-153). However, the observation that apoptotic effectors, such as caspases, not only play an important role but are vitally important to immune cell activation and proliferation came as somewhat of a surprise (154-156). Since then, it has become apparent that caspases are necessary for the differentiation of several cell types, including muscle (157). This thesis aimed to better characterize the biological effectors of apoptosis during skeletal muscle differentiation in order to determine a molecular cause for temporary caspase activation during this process. In this study we tested the hypothesis that activation of caspase-2 as well as mitochondrial fission contribute to transient increases in caspase-3 activity which are necessary for proper skeletal muscle differentiation.

Measurement of caspase-2 activity has not been performed in previous studies examining apoptotic signalling during skeletal muscle differentiation (50, 93-96, 98, 101, 102, 158-160). Our results indicated caspase-2 was actually activated first, starting 6 hours after differentiation was induced and reaching statistical significance in only 12 hours (Figure 4). In agreement with these earlier studies, caspase-3 activity in our C2C12 cells peaked early during the differentiation process: before most cell fusion events and the appearance of mature skeletal muscle markers.

Importantly, this response was observed after the induction of caspase-2 and was maintained at this increased level for 36 hours longer than that of caspase-2 (Figure 4). Caspase-3 is considered the primary executioner caspase (53), and most studies examining apoptotic regulation during skeletal muscles differentiation attribute their observations to alterations in the activity of this key enzyme. Several initiator caspases are known to exert their pro-apoptotic effects through activation of caspase-3 (53). While its identity has been debated (69) caspase-2 has been shown to initiate caspase-3 activation directly through pro-domain interactions (71) and indirectly through the mitochondria (63, 64). Our observation that caspase-2 activity was increased before caspase-3 during differentiation suggests it may be responsible for caspase-3 activation in this context. The other initiator caspases we measured, caspases-8 and -9, are well-characterized in their ability to activate caspase-3. However, while roles for caspases-8 and -9 have been identified by other researchers, activity levels of these enzymes were not observed to increase/change during skeletal muscle differentiation (Figure 4). These discrepancies may be due to methodological differences of caspase activity measurements (94, 95, 102) or the cell lineage used (50, 94, 101, 102, 158). As a result, it is possible that these two caspases are relevant in the differentiation of myogenic cell lines other than C2C12.

Although the several studies mentioned above implicate caspase activation as an important occurrence during skeletal muscle differentiation, canonical cellular causes of this, such as translocation of signalling molecules, association of enzyme activating platforms, and mitochondrial disturbance are not often investigated. Instead, most analyses involve comparing the attenuation of apoptotic signalling through chemical caspase inhibition to the effects caused by experimental increases/decreases to the expression of anti/pro-apoptotic genes. These results could be misleading as amplifications to protein expression may affect biological phenomena irrelevantly. Therefore, identifying the physiologically-relevant cause of caspase activation during skeletal muscle differentiation is warranted. Some investigations of typical pro-apoptotic signalling mechanisms have been performed, however results have been conflicting. Release of cytochrome c from the mitochondria has been detected by some (102, 159) but not others (95) during differentiation. In one study, this was shown to be dependent on the expression of PUMA, which was induced during differentiation (102). In fact, these researchers later indicated that PUMA can be upregulated by MyoD in this context (101). During apoptosis, cytochrome c release occurs in response to mitochondrial outer membrane permeablization (MOMP) (57). Assessment of mitochondrial membrane depolarization has been performed, however it was found to not decrease significantly during skeletal muscle differentiation, particularly compared to the change induced by staurosporine (95). Similar to Weyman and colleagues (101, 102), we observed increased whole-cell protein expression levels of PUMA, as well as Bax, after 1 day of differentiation (Figure 5). These two pro-apoptotic BH3 proteins are induced by p53 (77, 85, 161), so their increase is not surprising given the concomitant increases in p53's transcriptional activity typical of skeletal muscle differentiation (40, 114, 115). Furthermore, these increases occurred before/during the rise in caspase activity, supporting their potential role in the activation of caspases during skeletal muscle differentiation. Importantly, these elevated protein levels were temporary, as expression of both Bax and PUMA decreased after day 1.

The changes observed in anti-apoptotic proteins also support the contribution of canonical apoptotic signalling to the control of caspase activity during skeletal muscle differentiation. Caspase-3 activity was shown to progressively decrease between days 1.5 and 7, while levels of Bcl-2 reached their maximum on day 2 (Figure 4 & Figure 6). Transient expression of Bcl-2 during muscle differentiation has been observed previously and was necessary for formation of normal-sized myotubes (162). Bcl-2 possesses various, highly effective anti-apoptotic properties, and is known as “the prototype of anti-apoptotic proteins” (57). Since the time point of its highest expression coincides with the beginning of the decline in caspase-3 activity, it is possible that Bcl-2 is instigating this event. In accordance with this, overexpression of the anti-apoptotic Bcl-2 family member Bcl-$_{XL}$ led to a reduction in transient caspase activity and significantly delayed C2C12 differentiation as measured by cell fusion (95). However, these authors point out that expression of myogenin and myosin was not affected by increased Bcl-$_{XL}$ content, implying that attenuation of apoptotic signalling affected cell fusion independently of the appearance of myogenic markers (95). Another anti-apoptotic protein, the caspase inhibitor XIAP, decreased progressively during differentiation (Figure 6). This reduction implies it may be partly responsible for causing temporarily increased caspase activity. In fact, XIAP function has been shown to be more efficient in fully differentiated myotubes due to concurrent decreases in the pro-apoptotic factor Apaf-1 (163), which is normally required to overcome basal IAPs. Importantly, levels of the anti-apoptotic protein ARC were shown to dramatically rise during differentiation (Figure 6). While the members of Bcl-2 and IAP families are potent cellular protectors, their functions are more “down-stream” in the apoptotic signalling cascade, with Bcl-2 members exerting their function during mitochondrial pore formation and IAPs involved with directly inhibiting caspases (57). On the other hand, ARC can interrupt preceding apoptotic events by binding procaspases-2 and -8, preventing DISC formation, inhibiting Bax activation, and blocking PUMA and Bad (164). Hence, the drop in XIAP and subsequent rise in ARC during skeletal muscle differentiation suggests these proteins possibly contribute to caspase activity regulation during skeletal muscle differentiation, and that ARC presence may reduce the requirement for other anti-apoptotic proteins (165).

As mentioned above, the apoptosis-inducing functions of many proteins depend on their subcellular localization. Previous studies have not examined the mitochondrial localization of factors involved with pore formation such as Bax and Bcl-2. Consequently, we observed that protein levels of Bax rose 2.5-fold in mitochondrial-enriched subcellular fractions during the first 24 hours of differentiation (Figure 7). This reinforces its potential role in promoting mitochondrial-mediated apoptotic signalling. However, mitochondrial Bcl-2 levels increased in a similar manner, resulting in no change to the Bax:Bcl-2 ratio, considered a measure of apoptotic susceptibility (166), during early differentiation events (Figure 7). This indicates that although Bax expression is induced and localizes to mitochondrial early during differentiation, its pro-apoptotic functions are likely inhibited by concomitant increases in mitochondrial Bcl-2. Ultimately, mitochondria regulate apoptotic signalling through the release of several factors whose cellular localization prompts association with and activation of various apoptosis-inducing mechanisms (57). AIF release results in its nuclear translocation and leads to chromatin condensation and DNA fragmentation (60). Mitochondrial AIF release as measured in cytosolic-enriched subcellular fractions changed insignificantly during differentiation, however this response was highly variable (Figure 7). In these fractions, immunoblotting revealed the detection of two separate bands, one at ~60 kDa and one at ~57 kDa. Prior to its release, mitochondrial-bound AIF is cleaved, resulting in a pro-apoptotic, truncated form of AIF that migrates more quickly in SDS-PAGE (167, 168). Quantification of just the ~57 kDa band also did not show meaningful trends (*data not shown*); implying AIF is not involved during skeletal muscle differentiation. Correspondingly, primary skeletal muscle cultures from AIF-deficient mice displayed no reduction in proliferation and differentiation capacities (169). This suggests that caspase-independent mechanisms are not a major player in skeletal muscle differentiation;

however, other caspase-independent cell death factors such as EndoG and Omi1 have not been studied. Cytochrome c release is considered a key event of mitochondrial apoptotic signalling, coordinating with Apaf-1 and dATP in the cytosol to activate caspase-9 (51). Likewise, Smac release results in inactivation of endogenous inhibitors of apoptosis proteins (IAPs), such as XIAP, which bind to and inhibit caspases (59). Somewhat surprisingly, cytosolic levels of these two proteins dramatically and immediately decreased upon the induction of differentiation (Figure 7). After 12 hours, cytosolic Smac dropped 55%, reaching its lowest after 36 hours while cytosolic cytochrome c similarly decreased 67% in 12 hours, reaching its lowest level on day 2 (Figure 7). These two time points are, in fact, the same time points during which we observed the highest level of caspase-3 activity (Figure 4). Equally as surprising, the decreases in cytosolic Smac and cytochrome c were transient, as both returned to the levels observed on day 0 by day 5 (Figure 7). These results are contrary to our hypothesis, which was to detect more of these pro-apoptotic signalling molecules in the cytosol during early-differentiation events. Instead, it was during this time that cytosolic levels were lowest. Previously, Smac release was not detected in differentiating C2C12 cells as measured using immunoblotting of subcellular fractions (95). Likewise, these researchers were unable to detect cytochrome c in cytosolic fractions early during differentiation (>2 days) (95). However, a temporary increase in cytosolic cytochrome c has been observed by others (102). An important methodological variation made by these researchers is the inclusion of cells which die during differentiation into their biochemical analyses, a distinction they term apoptosis *associated* with skeletal muscle differentiation (101, 102, 158). Certainly, many myoblasts undergo cell death by apoptosis during this process, and this is a well-recognized and characterized phenomenon (158, 170, 171). We similarly observe accumulation of dead and/or dying cells during the first 1-2 days of C2C12 differentiation (qualitative observations). For our analyses, this cellular debris was washed away, as we were interested in the caspase activity and apoptotic cell signalling that occurred in the cells which remained healthy and continued to differentiate. Interestingly, cytochrome c-dependent caspase activity has been observed during the differentiation of other cell types such as lens epithelial cells (104) as well as macrophages (103). Importantly, a drop in mitochondrial membrane potential occurred prior to cytochrome c release during lens cell differentiation (104), a phenomenon which was not observed during skeletal muscle differentiation (95).

When considered together, these results support the theory that mitochondrial apoptotic signalling is not responsible for inducing caspase activity during skeletal muscle differentiation. A lack of mitochondrial release of pro-apoptotic factors, combined with absence of increased caspase-9 activity, suggests some other mechanism must be responsible for activating caspase-3 in this context. As previously mentioned, an interaction between caspase-2 and p53 was recently described (116). The transcriptional activity of p53 is temporarily increased and is required for proper skeletal muscle differentiation (40, 114, 115). In this newly proposed model, caspase-2 was shown to cleave and inactivate Mdm2, a protein responsible for p53 degradation (116). Since p53 is a transcription factor for the caspase-2 activator PIDD, this led to a positive-feedback loop resulting in increased p53 stability. In addition, caspase-2 promotes the function of PKCδ (172), a kinase with significant pro-apoptotic links (173), and proper activation of PKCδ is required for the induction of caspase-3 (174, 175). To examine the role of caspase-2 in myogenesis, we assessed the effects that inhibition of caspase-2 and caspase-3 has during skeletal muscle differentiation.

We first assessed the ability of C2C12 cells to undergo differentiation in the presence of a chemical inhibitor of caspase-3. Capase-3 inhibition reduced cell fusion and myotube formation in a concentration-dependent manner (Figure 9). Similarly, these changes were associated with delayed myosin expression as measured using immunoblotting (Figure 10). These observations indicate that caspase-3 activity is required for proper skeletal muscle differentiation. Myogenin was induced normally in C2C12 cells incubated with 30μM Ac-DEVD-CHO, although its expression did not drop later during differentiation (Figure 10). This supports a pro-myogenic effector role for caspase-3 activity, as myogenin expression was maintained in response to its inhibition. However, a higher concentration of caspase-3 inhibitor reduced maximal myogenin protein levels (Figure 10). These results suggest that caspase-3 activity may also be required for adequate myogenic gene expression. Curiously, even though the two concentrations of Ac-DEVD-CHO affected makers of skeletal muscle differentiation differently, each inhibited the activities of both caspases-2 and -3 equally (Figure 10). This would imply that caspase-2 is "downstream" of caspase-3. On the other hand, the typical mechanism of caspase-2 activation is dependent on its associated with the PIDDosome (68, 69). Caspase inhibitors such as Ac-DEVD-CHO are designed to be enzyme-specific based on preferred substrate cleavage sites. However, there is overlap in substrate preference between caspases (176), meaning that each concentration of Ac-DEVD-CHO we utilized could have also inhibited the enzymatic activity of caspase-2 as well as other caspases. With respect to caspase-3, it is possible that both concentrations inhibited enzyme activity at levels which were indistinguishable with our analysis method.

Several examinations have previously reported a supporting role for caspase-3 activity during skeletal muscle differentiation (50, 93, 95, 160). These studies conclude that adequate activation of caspase-3 is necessary for morphological changes and induction of pro-myogenic genes. While these researchers attribute functional relevance for caspase activity in the cells which achieve differentiation, others have concluded that the role of caspases during this process is simply to remove unnecessary cells through apoptosis (101, 102, 158, 159). Indeed, apoptosis is involved during the development of many tissues including skeletal muscle (170, 171, 177). However, Fernando et al (2002) observed activated caspase-3 in cells which became myosin-positive, and reported that administration of active caspase-3 was able to induce differentiation even in high-serum conditions (50). Furthermore, they showed that caspase-3-dependent cleavage of MST1 was required for proper expression of myogenic factors. Additionally, the activity of p53, a gene containing many pro-apoptotic associations, has been reported in cells which differentiate, but not myoblasts which undergo apoptosis during myogenesis (113). Regardless of its role, inhibition of caspase-3 has repeatedly been shown to impair skeletal muscle differentiation.

Based on our observation that caspase-2 is the first caspase to increase after inducing differentiation, we conducted a similar experiment by assessing the ability of C2C12 cells to undergo differentiation while in the presence of a chemical inhibitor of caspase-2. Capase-2 inhibition reduced cell fusion and myotube formation in a concentration-dependent manner (Figure 11). Likewise, these changes were associated with delayed myosin expression measured using immunoblotting (Figure 12). These observations indicate that caspase-2 activity is required for proper skeletal muscle differentiation and the effects of its inhibition are akin to those of caspase-3. Myogenin was induced normally in C2C12 cells incubated with 30μM Ac-VDVAD-CHO, although at 75μM this response was significantly inhibited (Figure 12). These results suggest that caspase-2 activity may also be required for adequate myogenic gene expression. Similar to caspase-3 inhibitor experiments, both concentrations of Ac-VDVAD-CHO affected markers of skeletal muscle differentiation differently, although each inhibited the activities of caspases-2 and -3 equally (Figure 12). As mentioned above, caspase inhibitors such as Ac-

VDVAD-CHO are designed to be enzyme-specific based on preferred substrate cleavage sites. However, due to the overlap in substrate preference between caspases (176), the higher concentration may have differentially affected myogenesis by inhibiting non-specific caspases. Administration of Ac-VDVAD-CHO did not reduce caspase-3 levels to the same level as Ac-DEVD-CHO, but did result in similar reductions to myogenic development. Therefore, the effects that Ac-VDVAD-CHO had on differentiation are due to its distinctive influences on the activation of caspase-2 and/or possibly other caspases.

The most logical role for caspase-2 during skeletal muscle differentiation is in the activation of effector caspases such as caspase-3. However, its best understood mechanism of apoptotic induction is through Bid-cleavage-dependent mitochondrial permeablization and subsequent release of apoptotic factors such as AIF, Smac, and cytochrome c (63, 64). Our examination of mitochondrial apoptotic signalling indicates this pathway is not active during differentiation. Furthermore, although caspase-2 shares structural homology and activation mechanisms with other initiator caspases, unlike caspases-8 and -9 it does not cleave effector procaspases, therefore excluding it from the caspase cascade (63). Consequently, even if a novel caspase-2 activation method was identified, it is unlikely that this would lead to direct activation of other caspases. Interestingly, caspase-2 has been shown to contribute to cell cycle arrest in response to DNA damage (100, 178). Activation of p53 is a well-known response to DNA damage (179, 180), and as mentioned above, p53's regulatory and transcriptional activities are required for skeletal muscle differentiation (40, 113-115). Due to the presence of a positive feedback loop between caspase-2 activity and p53 (116), its purpose may be to temporarily maintain p53 function early during differentiation. Although this is a possibility, a comprehensive examination of caspase-2 cleavage substrates has not been performed, so its function during skeletal muscle differentiation may be activating/inactivating a yet unidentified factor.

One of our original hypotheses was that mitochondrial fission is partly responsible for caspase activation during skeletal muscle differentiation, as it is known to contribute to the release of pro-apoptotic factors into the cytosol (129). Although myotube development is associated with production of large mitochondrial networks (139, 140), we postulated that fission could be increased transiently during this process. Results showed that protein expression of Drp1 increased, while Mfn2 decreased during differentiation (Figure 8). However, these changes were progressive, and are consistent with a “pro-fission” phenotype upon terminal differentiation instead of prior to caspase activity. As Drp1 must translocate to the mitochondria to induce fission (118), immunoblotting in mitochondrial-enriched fractions was also performed. Here, a transient mitochondrial localization of Drp1 occurred mid-way during the skeletal muscle differentiation timeline (Figure 8). These results somewhat contradict previous examinations of mitochondrial dynamics during myogenesis. Zorzano and colleagues (139) observed increased Mfn2 levels in myotubes compared to proliferative myoblasts. Furthermore, this study showed that Mfn2 was required to maintain mitochondrial network architecture and metabolic function (139). However, the effect that alterations to Mfn2 levels had on the ability to differentiate was not examined (139). Building on this work, other researchers demonstrated that nitric oxide (NO)-induced inhibition of fission must occur during myogenesis in order to generate large mitochondrial networks typical of myotubes (140). Nitric oxide synthase (NOS) activity does in fact transiently increase and is required for myotube production during skeletal muscle differentiation (181). In this recent study, researchers demonstrated that NO inhibited Drp1 through G-kinase-dependant phosphorylation (140). However, Drp1 activity is both positively

(120, 182) and negatively (137) regulation by phosphorylation. Furthermore, the primary myocytes used in this study expressed myosin on day 0 (140), and as a result, these observations may represent changes occurring later during the differentiation timeline than the transient increase in fission that we observed. In addition, NO has commonly been shown to actually induce mitochondrial fission in neuronal cells (183, 184), and functional Drp1 is necessary for proper neuronal development (119, 185). Given our observation that mitochondrial-mediated apoptotic signalling is not responsible for activating caspases during skeletal muscle differentiation, the involvement of mitochondrial fission during this process must lie someplace else. Instead, as increased mitochondrial Drp1 was observed after the peak in caspase activity, it is more likely responsible for mitochondrial transport during the extreme changes to cell morphology which occur during the transition from myoblast to myotube. Similar conclusions were realized during the examination of neuronal development in the absence of Drp1. While mitochondria were still formed and maintained a level of metabolic function, the network did not extend into dendritic processes, resulting in blunted synapse and neurite formation (119).

No work has been published regarding the relationship between apoptotic signalling and mitochondrial fission during skeletal muscle differentiation. To examine this, we inhibited mitochondrial fission using mdivi-1, a chemical inhibitor of Drp1 function (141). As with caspase inhibitors, the effects of mdivi-1 administration on myogenesis were dose-dependent. At a low concentration, a reduction in myotube development and cell fusion was observed, while these two events were almost completely abolished at the higher concentration (Figure 13). Particularly at 50μM mdivi-1, myosin-positive single-nucleated cells were found instead of myotubes. These morphological observations imply that mitochondrial fission is required for myotube formation. Due to the pro-apoptotic association of mitochondrial fission, our hypothesis was that transiently increased fission contributes to the release of apoptosis-inducing factors and hence to the activation of caspases. Contrary to this, inhibition of mitochondrial fission resulted in dramatic dose-dependent increases in the activities of caspases-2 and -3 during differentiation (Figure 14A). Almost all previous research supports a pro-apoptotic role for mitochondrial fission (129), so this observation was rather surprising. At first glance, this seems due to the toxic nature of the chemical. However, mdivi-1 administration alone did not produce obvious apoptotic changes during concentration tests (Appendix Figure 2), the total number of adhered cells (from fusion index calculations) and dead cells present in culture media (*data not shown*) were not different between control and 20μM mdivi-1 groups, and, importantly, the increase in caspase activity remained transient. If in fact mdivi-1 was inducing caspase-dependent apoptosis due to acute toxicity, we should have observed more dead cells, less adhered cells, and caspase activity would have increased until all myoblasts were eliminated from culture dishes. Instead, the number of dead and adhered cells was similar between control and 20μM groups, and caspase activities remained transient in both mdivi-1 treatment conditions. It appears as though mdivi-1 prevented the function of caspases during differentiation, as if fission was "down-stream", and caspases were activated to a higher degree in response. This paradigm is difficult to reconcile however, as no mechanism of caspase-induced changes to mitochondrial morphology have been observed. The most likely explanation is that long-term administration of mdivi-1 resulted in stress-induced apoptotic signalling due to the prolonged inability of these cells to undergo proper mitochondrial fission. Although it is well documented that inhibition of mitochondrial fission reduces the short-term apoptotic response to an appropriate stressor (129, (Appendix Figure 2)), there is evidence that prolonged inhibition leads to increased cell stress and apoptosis (186-188). Again, this explanation does not clarify why the increases in caspase activity we observed with mdivi-1 treatment occurred in an identical timeline to that observed in control cells.

Regardless, these morphological observations and alterations to caspase activity were associated with changes in the expression pattern of myogenic markers. While myogenin expression increased slightly in myoblasts given 20μM mdivi-1, its induction was delayed and the peak expression levels were significantly less than in control cells (Figure 14). Similarly, this treatment led to reduced myosin levels once control cells fully differentiated (Figure 14), which, combined with decreased cell fusion, indicates that fission is required for regulating changes to cell morphology and the expression of myogenic-specific proteins. A high concentration of mdivi-1 completely prevented myogenin and myosin levels from increasing (Figure 14), supporting evidence that changes to mitochondrial morphology provide feedback in order to regulate transcription and complete expression of skeletal muscle-specific genes (189, 190). However, given the unexpected observation that inhibition of mitochondrial fission resulted in dramatically increased transient caspase activity, it is unclear whether mdivi-1 administration influenced myogenesis through its effects on mitochondrial morphology, or whether myogenic development decreased in response to excessive proteolytic activity during early differentiation events.

These results provide evidence that the functional relevance of mitochondrial fission during skeletal muscle differentiation is not in the promotion of apoptotic signalling, but we suggest it likely contributes to morphological changes to the mitochondrial network associated with myotube formation. As mentioned above, researchers examining neuronal differentiation in the absence of Drp1 came to a similar conclusion (119). Given the remarkable changes to cell morphology which occur during both muscle and neuron development, this is not surprising.

There are several mechanisms controlling mitochondrial fission which may be relevant during skeletal muscle differentiation. Regulation of Drp1 translocation has frequently been examined, and one important mediator of this event is its phosphorylation status. Phosphorylation of Drp1 by CamK (182), cdk1/cyclin B (120) and dephosphorylation by calcineurin (136, 137), have been shown to increase mitochondrial fission, while phosphorylation by PKA (137) has been shown to inhibit mitochondrial fission. These studies demonstrate that fission can be induced during cell proliferation and in response to Ca^{2+} signalling. As differentiation is associated with a withdrawal from cell cycle, this mechanisms is likely not as relevant. However, the ability of calcium to act as both a transcription factor and enzyme regulator is vitally important during skeletal muscle differentiation (191-193). In accordance with this, reduced cellular calcium (194) and inhibition of calcineurin activity (195, 196) restricted myotube development, although it is unknown if these interventions affected mitochondrial morphology. In addition to Ca^{2+}-induced mitochondrial fission, experiments have also shown that cytosolic calcium handling is affected during manipulations to mitochondrial morphology (197, 198). Therefore, it is possible that mdivi-1 treatment led to altered calcineurin/NFAT signalling, proper activation of which is necessary for myogenin expression and skeletal muscle differentiation (199, 200).

Summary and Conclusions

This thesis serves as the most comprehensive examination of apoptotic signalling during skeletal muscle differentiation. Several novel findings were observed: 1) caspase-2 is activated very early during the differentiation process, 2) the mitochondrial Bax:Bcl-2 ratio does not change during early differentiation events, 3) cytosolic Smac and cytochrome c levels decrease prior to and during the spike in caspase activity, 4) differentiation is associated with progressively increased and decreased expression of the anti-apoptotic proteins ARC and XIAP, respectively, and 5) Drp1 transiently locates to the mitochondria after caspase activity peaks. When considered together, these results provide evidence supporting the notion that mitochondrial-mediated apoptotic events are not responsible for activating caspases during skeletal muscle differentiation.

This study is also the first to evaluate contribution of caspase-2 activity to skeletal muscle differentiation. In response to pharmacological inhibition of caspase-2, cells displayed reduced myotube formation and markers of terminal differentiation. Importantly, these myogenic changes were similar to those observed in response to chemical inhibition of caspase-3.

Finally, we demonstrate that mitochondrial fission is necessary for skeletal muscle differentiation. Although this agrees with our hypothesis, we initially thought this would be due to fission promoting the release of pro-apoptotic factors into the cytosol. Instead, it appears that fission may be more important after caspase activity in the differentiation timeline, leading us to the conclusion that it participates in altering mitochondrial network morphology. Possibly, these changes may affect skeletal muscle differentiation through mitochondrial retrograde signalling and/or Ca^{2+} myogenic transcriptional gene regulation. Regardless, these data highlight another physiological function that requires specific control of mitochondrial dynamics.

Limitations

As already mentioned, the chemicals used in this study to inhibit caspase activity such as Ac-DEVD-CHO and Ac-VDVAD-CHO are designed to be enzyme-specific based on preferred substrate cleavage sites. However, due to the overlap in substrate specificity between caspases, these chemicals are not perfectly exclusive for their respective enzyme. As a result, the effects of their administration may be due to influences on additional, un-intended caspases.

A distinction should be made between what was examined in this study and apoptosis *associated* with myoblast differentiation. As discussed above, several researchers attribute the transient increase in caspase activity observed during skeletal muscle differentiation to early-apoptotic events occurring in cells which are adherent but currently undergoing cell death processes. Although care was taken during cell isolations to remove dead and/or dying cells by washing culture dishes with PBS, the contribution of these destined-to-die cells cannot be excluded. Even so, inhibition of caspases has repeatedly been shown to impair skeletal muscle differentiation, supporting a role for apoptotic control of this process. Furthermore, caspase-dependent activation of pro-myogenic factors has been established, and the consensus is that these enzymes have regulatory functions during differentiation and proliferation of several other cell types through their interactions with proteins involved with cell cycle, cytokine maturation, cell adhesion, immunity, G-protein activation, etc. (54, 157).

While we did not detect involvement of initiator caspases-8 and -9 during C2C12 differentiation, other researchers using similar methods have indicated contribution of these enzymes during the differentiation of other skeletal myogenic cell lines such as L6E9 and 23A2. Therefore, it is possible that these caspases have relevance in skeletal muscle culture models other than C2C12. Additionally, although immortalized myoblasts capable of *in vitro* differentiation allow examination of this process under controlled conditions, the mechanisms controlling myogenesis *in vivo* may be different than those observed during cell culture experiments. In agreement with this, experiments performed with primary cell cultures also display conflicting findings (50).

Future Directions

The results of this thesis suggest mitochondrial pro-apoptotic signalling does not contribute to caspase activation during skeletal muscle differentiation. Although evidence for canonical mitochondrial release of pro-apoptotic factors has been observed during apoptosis associated with myoblast differentiation, these events have never been confirmed in differentiating cells. The activation of caspases from other apoptotic pathways has also been detected, but similar to mitochondrial-mediated mechanisms, causes of their activation have not. As a result, how caspases become activated in this context is still unknown. While complex signalling pathways such as NF-κB, p38/MAPK, and JNK are implicated in controlling the fate of many cells including skeletal muscle, their effects on apoptotic signalling must still culminate in caspase activation. Therefore, although these upstream signalling mechanisms are likely responsible for regulating the decision between cell differentiation and cell death, there are only so many ways to activate caspases, and one of them must be occurring during skeletal muscle differentiation.

Research has shown that some features typical of apoptosis (actin fiber dis/re-assembly, extracellular phosohpatidylserine expose) also occur during skeletal myogenesis, indicating that caspase targets may overlap during these two processes. Furthermore, although some direct pro-myogenic roles for caspases have been identified, definitive, indispensable functions for caspase-dependent cleavage events have not been thoroughly investigated. Therefore, a more complete examination of the exact substrates cleaved by caspases and their purpose during skeletal muscle differentiation is warranted.

Due to the potential non-specific effects that chemical caspase inhibition involves, an assessment of caspase-2 function during myogenesis through genetic manipulation of its expression would provide more concrete evidence of its role. Although *caspase-2* null mice seem to develop normally (201), the effects of its deficiency during skeletal muscle differentiation may be concealed due to redundancies in caspase substrate specificity.

Finally, we reasoned that anti-apoptotic proteins such as Bcl-2 and ARC were responsible for ensuring caspase activity remained transient. This hypothesis could be examined by inhibiting the function or expression of these factors and observing the effects that this has on caspases and the extent of differentiation.

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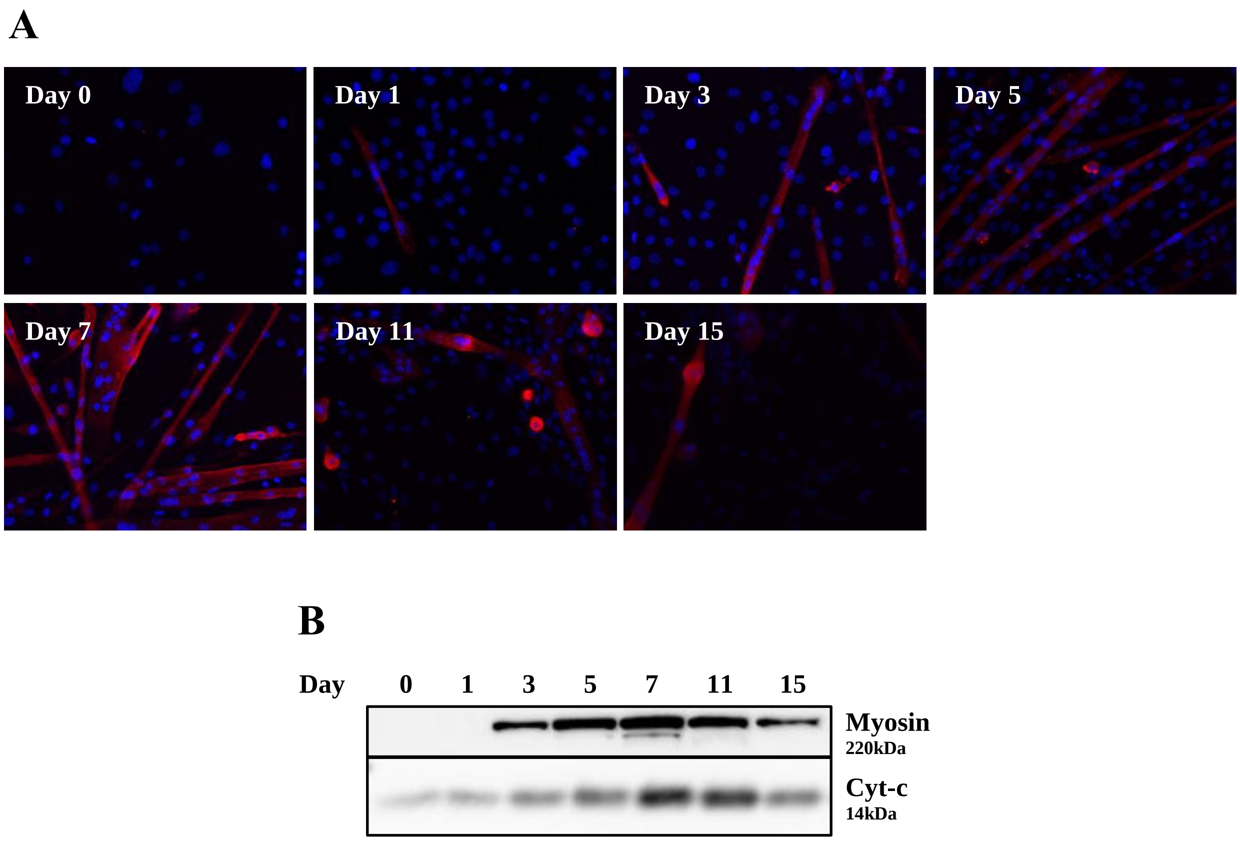
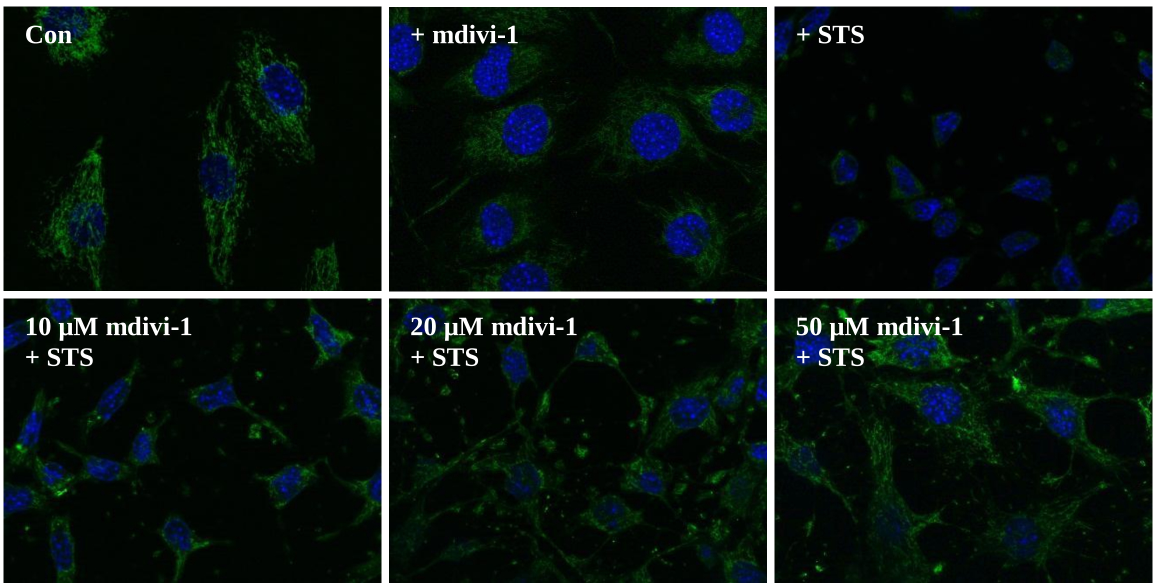
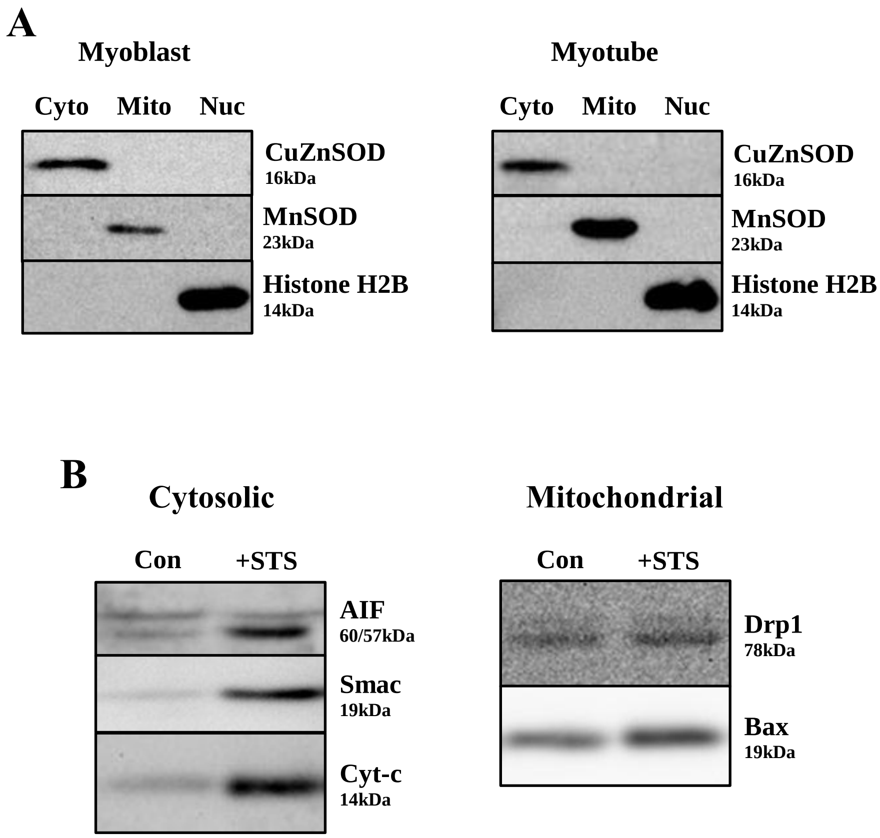
Appendix



Appendix Figure 1: Determination of appropriate in vitro differentiation timeline. C2C12 cells were induced to differentiate upon reaching 70-80% confluence and were harvested after spending indicated amounts of time in differentiation media. As can be seen in **A)**, myotube development increases until day 7, but drops significantly thereafter. **B)** These morphological changes were associated with progressive increases in myosin and mitochondrial content as indicated by cytochrome c expression until day 7, after which levels of both decline.



Appendix Figure 2: Determination of working mdivi-1 concentrations. The ability of mdivi-1 to inhibit mitochondrial fission resulting from an apoptotic stress (2µM staurosporine, STS, for 2 hours) was tested by incubating C2C12 cells with increasing concentrations of mdivi-1. Mitochondria were visualized using MitoTracker and nuclei with DAPI. Incubation with mdivi-1 alone resulted in elongated networks of mitochondria, whereas incubation in STS alone resulted in nuclear condensation and cell blebbing typical of apoptotic cell death. As can be seen in lower panels, mitochondrial fragmentation and apoptotic changes to cell morphology induced with STS were progressively inhibited by increasing concentrations of mdivi-1.



Appendix Figure 3: Validity of subcellular fractionation procedure to detect appropriate molecular response to apoptotic stress. A) Fraction purity confirmation in myoblasts and myotubes. **B)** C2C12 cells were left untreated (Con) or incubated with 2μM staurosporine for 2 hours (+STS) and then subjected to subcellular fractionation. Mitochondrial release of AIF, Smac, and cytochrome c is apparent in STS-treated cells. Likewise, STS induced mitochondrial translocation of Drp1 and Bax.