Cleveland State University
EngagedScholarship@CSU

2009

Myf5 Does not Induce Apoptosis in Skeletal Myoblasts but is Regulated by Oncogenic Ras Expression

Alexander Phillip Talarico
Cleveland State University

MYF5 DOES NOT INDUCE APOPTOSIS IN SKELETAL MYOBLASTS BUT IS

REGULATED BY ONCOGENIC RAS EXPRESSION

ALEXANDER P. TALARICO

Bachelor Of Science

John Carroll University, Cleveland, Ohio

May, 2006

submitted in partial fulfillment of requirements for the degree

MASTER OF SCIENCE IN BIOLOGY

at the

Cleveland State University

December, 2008

This thesis has been approved for

the Department of Biological, Geological, and Environmental Sciences

and the College of Graduate Studies by

_____ Date: _____ .
Crystal M. Weyman,
Committee Chairperson, Department of BGES,
Cleveland State University

_____ Date: _____ .
Anton A. Komar,
Department of BGES,
Cleveland State University

_____ Date: _____ .
Barsanjit Mazumder,
Department of BGES,
Cleveland State University

ACKNOWLEDGEMENTS

success for you. Auntie Toto, where would we be without your phone conversations in Persian, exuberant laughter, and mispronunciation of English words? You brought a lot of life to the lab and provided plenty of material for Kevin's incessant wisecracking. Kevin, you've been a good friend (and workout buddy). One way to encourage success is to make failure unpalatable. Thanks for thickening my skin and helping me to care a lot less about what people think. I also owe my renewed interest in weightlifting to you, so thanks for the motivation, and it's been great to have someone at work who likes indie rock to talk to. To Terri, thanks for teaching me several techniques and being a resource when I had questions. At least now if I become a research doctor I'll know what I'm doing in the lab. To Greg, I am 99% sure that liquid caffeine courses through your veins instead of blood. You were a lot of fun in lab and in class. Best of luck with your new baby. Maybe I'll see you at Frankie's, where you will finally give me that free pizza you keep promising me. Finally, to JR, thank you for playing peacemaker so many times. It doesn't go unnoticed. The way you handle your life is an inspiration. A lot of people would fold up under the pressure, but you for the most part take it in stride (Cue 'Under Pressure' by Queen as background music).

Special thanks to my parents who have encouraged me from day one and never once told me I couldn't do what I thought I was capable of doing. Thank you so much for believing in me, I love you both. You have always sacrificed for me, and I can't thank you enough.

Finally, thank you to Katie for all the support and warmth you have offered me for these past two years. Research can be emotionally taxing when things don't go well and you always offered plenty of encouragement if things were tough. You too have been an integral part of my success, and I thank you with all my heart.

To the rest of my close friends: "We did it!"

MYF5 DOES NOT INDUCE APOPTOSIS IN SKELETAL MYOBLASTS BUT IS

REGULATED BY ONCOGENIC RAS EXPRESSION

ALEXANDER P. TALARICO

ABSTRACT

Skeletal myogenesis is initiated by a family of muscle regulatory factors (MRFs) composed of the transcription factors MyoD, Myf5, MRF4, and myogenin. Of these, MyoD was the first discovered, is the most studied, and plays a well-characterized role in skeletal myoblast determination and differentiation. Our laboratory has also discovered that MyoD plays an important role in the apoptotic process that occurs in a subpopulation of myoblasts induced to differentiate. Expression of oncogenic Ras is known to inhibit myoblast differentiation and to decrease the expression of MyoD mRNA and protein. Our laboratory was the first to report that expression of oncogenic Ras also blocks the apoptotic process. While it is well known that MyoD and Myf5 function somewhat redundantly with respect to their role in the differentiation process, neither the role of Myf5 in the apoptotic process nor the regulation of Myf5 by oncogenic Ras has been investigated. Herein we report that, unlike MyoD, Myf5 is not sufficient to induce apoptosis. Further, like MyoD, Myf5 expression is decreased in both myoblasts and fibroblasts expressing oncogenic Ras. Additionally, we report that the mechanism of this inhibition is not a consequence of reducing Myf5 mRNA levels.

TABLE OF CONTENTS

LIST OF FIGURES

Figure.

CHAPTER I

INTRODUCTION

1.1 The Skeletal Myoblast Model System

The skeletal muscle system is a useful model for elucidating the cell signaling pathways regulating cell differentiation and apoptosis. During development, myoblasts are maintained in an actively proliferative state by circulating mitogens such as fibroblast growth factor (FGF) (Olson et al. 1985). Myoblast migration results in a reduction of the local concentration of such mitogens. Therefore, skeletal myoblasts cultured *in vitro* can be induced to undergo differentiation by mitogen withdrawal (Konigsberg 1971). Specification (determination) to the myogenic lineage and eventual differentiation depends on a group of basic helix-loop-helix (bHLH) transcription factors known as muscle regulatory factors (MRFs). These factors consist of MyoD, Myf5, MRF4, and myogenin (Rudnicki and Jaenisch 1995). In the presence of mitogens, these factors are prevented from binding DNA or acting through the interaction with Id

and/or histone deacetylases (HDACs) (Olson 1992). In the absence of mitogens, Id is degraded and histone acetyltransferases (HATs) are recruited (Mal et al. 2001). Differentiation is a stepwise process consisting of cell cycle exit, expression of muscle specific genes such as myogenin and myosin heavy chain, and fusion of myoblasts into multinucleate myotubes (Andres and Walsh 1996). The most frequently used myoblast cell lines are either 23A2 myoblasts (derived from 10T½ fibroblasts isolated from C3H mice) or C2C12 myoblasts (also isolated from C3H mice).

1.2 Expression And Role Of MRFs During Development

During development, Myf5 expression is typically restricted to the epaxial myotome (and transiently in some preneurons) and is signaled for by Sonic Hedgehog (Shh) and Wingless-type (Wnt) produced by the notochord and neural tube (respectively). Conversely, MyoD expression is found in the hypaxial myotome (Tapscott 2005). In both cases, MyoD or Myf5 activates expression of myogenin during differentiation. MRF4 expression is known to be induced late in the differentiation process. However, the mechanism controlling MRF4 expression remains unclear. Myf5 is also capable of inducing MyoD expression, and MyoD can positively regulate its own expression and can negatively regulate Myf5 expression (Emerson 1993). However, in differentiated myotubes, while Myf5 protein levels diminish, MyoD and myogenin continue to be expressed while MRF4 is expressed (Chang et al. 2007).

Each of the MRFs serves a specific role in development. Phenotypes of gene knockouts of these regulatory factors gives insight as to the importance of each MRF. MyoD knockout mice are phenotypically normal (Emerson 1993). Myf5 null mice are delayed in differentiation, display impaired muscle regeneration, and show an ongoing slow myopathy, but are otherwise normal (Gayraud-Morel et al. 2007). A knockout of MRF4 has proved problematic as different targeting vectors differentially affect the expression of Myf5 due to their overlapping enhancers (Chang et al. 2004). Myogenin knockout mice exhibit a lack of skeletal muscle at birth despite the presence of determined myoblasts, indicating myogenin's importance for terminal differentiation but not specification (Arnold and Braun 1996). Complete absence of both determined myoblasts and skeletal muscle is observed only in a MyoD/Myf5/MRF4 triple knockout mouse indicating that each is capable of specifying the muscle lineage (Ustanina et al. 2007). Thus, MRF4 seems to serve a redundant function to MyoD or Myf5 although during normal development MRF4 is only expressed post-differentiation (Gayraud-Morel et al. 2004). Thus, MyoD and/or Myf5 are considered to be the critical regulators of skeletal myoblast specification during development.

1.3 Expression And Role Of the MRFs During Regeneration

Satellite cells are adult stem cells which exist at the periphery of mature skeletal muscle (Mauro 1961). Normally quiescent, they are typically activated in response to muscle injury, resulting first in proliferation as myoblasts and ending in myogenic differentiation and subsequent augmentation of existing muscle fibers (Bodine-Fowler 1994; Schultz and McCormick 1994). These cells express MyoD and/or Myf5 when cultured in growth media (GM) (Yablonka-Reuveni et al. 2008). Therefore, most established myoblast cell lines such as C2C12 myoblasts are thus derived from these progenitors. In GM, 23A2 skeletal myoblasts express only MyoD, while C2C12 myoblasts express both MyoD and Myf5. MyoD knockout satellite cells upregulate Myf5, but exhibit a reduced ability to differentiate (Rudnicki et al. 1999; Montarras et al. 2000). Myf5 knockout satellite cells also display inhibited differentiation (Gayraud-Morel et al. 2007).

1.4 Coordinated Regulation Of Differentiation And Apoptosis

This laboratory, along with others, has previously reported that mitogen withdrawal actually results in two mutually exclusive physiological endpoints; 70% of cells in a population undergo differentiation while 30% undergo apoptosis (Wang and Walsh 1996). Apoptosis is a crucial component of skeletal myogenesis during development and regeneration and is thought to function to remove excess myoblasts. Defective apoptosis can contribute to malignancies

such as rhabdomyosarcoma (a cancer of skeletal muscle origin) (Scholl et al. 2000). Conversely, excessive apoptosis can worsen degenerative disorders such as spinal muscular atrophies, cachexia (muscle wasting), or muscular dystrophies and adds to ischemia-reperfusion injury following myocardial infarction (Weyman et al. 2002; Miller and Girgenrath 2006).

Cultures of both primary and established myoblast cell lines (23A2, C2C12) have been noted to undergo apoptosis when cultured in differentiation media (DM) (Shen et al. 2003). Interestingly, our laboratory has determined that 10T½ fibroblasts (from which 23A2 myoblasts are derived) quiesce rather than undergo either differentiation or apoptosis when cultured in DM (Weyman, personal observation). Since fibroblasts do not express any of the MRFs, one possibility was that MyoD was facilitating both differentiation and apoptosis. Others have reported that expression of either MyoD or Myf5 is sufficient to induce differentiation as monitored by the expression of Myosin Heavy Chain (MHC). Our laboratory subsequently determined that ectopic expression of MyoD is sufficient to induce apoptosis in 10T½ fibroblasts. Further, myoblasts silenced for MyoD expression are defective for both differentiation and apoptosis. However, the ability of Myf5 to induce apoptosis in either myoblasts or fibroblasts is, until now, untested.

1.5 The Role Of Oncogenic Ras In Skeletal Myoblasts

Cancer is a disease wherein cells proliferate uncontrollably due to defective differentiation and/or apoptosis. The formation of tumors relies on the mutation of certain genes (oncogenes and/or tumor suppresor genes) encoding the regulatory proteins that ensure that these processes work correctly (Spandidos 1986). Approximately 30% of all human malignancies and 50% of rhabdomyosarcomas contain a mutant version of the *Ras* gene (Linardic and Counter 2008). Ras is a GTPase. In response to growth factor signaling, normal Ras releases bound GDP and binds GTP. Ras*GTP initiates several signaling cascades. Ras*GTP is then inactivated via the GTPase activating protein (GAP). Oncogenic Ras protein is indifferent to GAP activity and thus remains in the GTP bound, constitutively active conformation. Others have shown that the expression of oncogenic Ras downregulates the expression of MyoD mRNA and protein and inhibits differentiation (Konieczny, et al. 1989). Our laboratory has confirmed that 23A2 myoblasts expressing an oncogenic constitutively active version of Ras (A2:G12V H-Ras) are defective for both differentiation and apoptosis (Weyman and Wolfman 1997).

The inhibition of differentiation and apoptosis seen in A2:G12V H-Ras myoblasts is likely a consequence of Ras signaling decreasing MyoD expression. The mechanism responsible for downregulation of MyoD expression by

oncogenic Ras signaling is unknown. Likewise, the effect of oncogenic Ras signaling on Myf5 expression has not been explored until now.

CHAPTER II

MATERIALS AND METHODS

2.1 Cells And Cell Culture

23A2 myoblasts are derived from 10T½ fibroblasts (Pinney et al. 1988). The growth of 10T½ fibroblasts and the derivative expressing G12V:H-Ras (10T1/2;G12V H-Ras), as well as the growth and differentiation properties of 23A2 myoblasts and the 23A2 derivative expressing G12V:H-Ras (A2:G12V;H-Ras myoblasts) have been reported previously. All cells were cultured on gelatin-coated plates and maintained in growth medium (GM), which consists of basal modified Eagle's medium (BME), 10% fetal bovine serum (FBS), and a 1% combination of 10,000 I.U./ml penicillin and 10,000 µg/ml streptomycin (1% P/S). Differentiation was induced by switching cells from growth medium to differentiation medium (DM), which consists of BME, 1% P/S and 0% FBS. Cycloheximide was dissolved in 1ml of ethanol to give a final concentration of 10mg/ml. Cells were treated with 1µl/100mm culture dish to inhibit translation.

2.2 Transient Transfection And B-galactosidase Assay

Equal cell numbers were plated and the next day transfected using Lipofectamine Plus per manufacturer's instructions (Invitrogen). Following overnight culture in GM, cells were washed three times in 1X PBS (phosphate-buffered saline pH 7.4) and fixed in 2.5% glutaraldehyde solution for five minutes at room temperature. The cells were then incubated with X-Gal solution (.1M potassium ferricyanide, .1M potassium ferrocyanide, 1M magnesium chloride, 40mg/ml X-gal) overnight at 37°C in the absence of carbon dioxide and the presence of B-gal activity was detected by counting stained vs. unstained cells.

2.3 Immunoblot Analysis

Samples were rinsed three times in cold 1x phosphate buffered saline (PBS) solution and then lysed in p21 lysis buffer (200mM sucrose, 0.1mm EDTA, 4mM MgCl$_2$, 20mM MOPS pH 7.4, 0.001 percent DNAse, 1 M phenylmethylsulfonyl fluoride, and 50 ug/ml each of leupeptin, pepstatin, and aprotinin) containing 1% CHAPS. Following protein determination, lysates (100 µg of total cellular lysate for MHC and MYF5) were denatured in 5x sample buffer (10% SDS, 50% glycerol, 10% 2-mercaptoethanol, pH 6.8) and electrophoresed through denaturing polyacrylamide gels. Following SDS polyacrylamide gel electrophoresis (SDS-PAGE), samples were transferred electrophoretically for

four amp hours to Hybond-P polyvinylidene difluoride membranes in transfer buffer containing 80% methanol and 1g/L SDS. Membranes were blocked for one hour in 1xTBS/0.1%NP40 with 10% newborn calf serum and 5% dry milk. The following primary antibodies were incubated with the appropriate membranes: mouse monoclonal anti-MHC antibody (MF20 ref), rabbit polyclonal anti-Myf5 antibody (Santa Cruz) diluted 1:1000, and mouse monoclonal anti-actin (Sigma) diluted 1:30,000. Appropriate HRP-conjugated secondary antibodies, each diluted 1:1000, were incubated with the membranes for one hour. After each incubation with antibody and prior to the addition of chemiluminescent substrate, membranes were washed five times in 1xTBS (tris-buffered saline pH 7.4) with 1% NP-40. Membranes were washed a final time with 1x TBS without NP-40.   Membranes were then incubated with SuperSignal West Pico Chemiluminescent Substrate (Pierce) for 60 seconds and bands were visualized using

Kodak Scientific Imaging Film.

2.4 Cytosolic Nucleosome ELISA

The presence of cytosolic nucleosomes indicative of DNA fragmentation and nuclear membrane disruption was used as a marker for apoptosis. Myoblasts were plated at equal density and the next day switched to fresh GM or DM. Cytosolic nucleosomes were measured using the Cell Death Detection ELISA Plus Kit (Roche Diagnostics) per manufacturer's instructions. Briefly,

attached cells were rinsed in PBS and then lysed by incubation in 500µL cell lysis buffer from the kit on a rocker for 30 minutes at room temperature. Twenty microliters of each sample was transferred to a 96-well, flat-bottomed, streptavidin-coated microtiter plate. Seventy-two microliters of 1x incubation buffer from the kit, and 4µL each of biotin conjugated anti-histone antibody and peroxidase conjugated anti-DNA antibody was added to the lysate in the microtiter plate and incubated at 4°C overnight. Following three washes with 300µL of incubation buffer, the presence of cytosolic nucleosomes was measured by adding 100µL of the substrate, ABTS. Absorbance at 405nm was measured at 60 second intervals using a spectrophotometer. Values were taken from the time point where all samples were within the linear range of the assay for each individual experiment.

2.5 Reverse Transcripts And Realtime PCR

Myf5 cDNA was transcribed using SuperScript III (Invitrogen) used per the manufacturer's instructions. The primer sequences used were FMyf5 (GAAGGTCAACCAGGCTTTCG) and RMyf5 (GTACTGCTCTTTCTGGACC), yielding a 252bp fragment. One other primer pair was used, giving similar results. Sample products were normalized to GAPDH as an internal standard. – RTs were used as a control for the presence of genomic DNA.

CHAPTER III

RESULTS

3.1 Myf5 Is Capable Of Inducing Differentiation, But Not Apoptosis, When Ectopically Expressed

We first compared the level of ectopic Myf5 expression in 10T½ fibroblasts to the endogenous levels in C2C12 skeletal myoblasts. Following transient transfection with various amounts of pcMyf5 and Western analysis for Myf5, we documented that 10T½ fibroblasts expressed Myf5 protein in a dose-dependent fashion (Fig 1). Transfection with 250ng per 10^5 cells yielded expression of Myf5 most comparable to the endogenous Myf5 levels seen in C2C12 myoblasts.

To confirm the functionality of Myf5 in our cells, we sought to recapitulate the capability of ectopic expression of Myf5 to induce differentiation in fibroblasts which had been reported by others. Following transient transfection with pcMyf5, 10T½ fibroblasts were assayed for Myf5 expression via Western analysis (Fig 2A). Parallel cultures were then switched from growth media
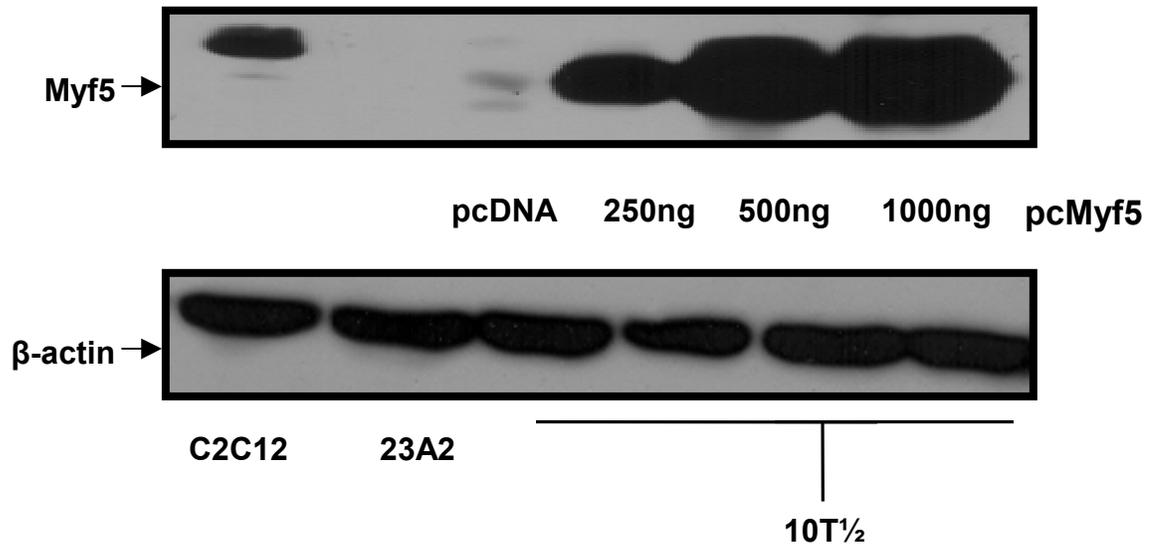
FIGURE 1. Ectopic Myf5 Expression In 10T½ Fibroblasts. Equal numbers of cells were plated and then transiently transfected with either pcDNA or pcMyf5 the next day. Following 24 h of culture in GM, lysates were made from the transfected cells. Western analysis to detect Myf5, with actin functioning as a loading and transfer control, was performed.

FIGURE 2. Ectopic Myf5 Expression Is Sufficient To Induce Differentiation In Fibroblasts. In (A), equal cell numbers were plated and then transiently transfected with either pcDNA or pcMyf5 the next day. Following 24 h of culture in GM, lysates were made from the transfected fibroblasts. Western analysis to detect Myf5, with actin functioning as a loading and transfer control, was performed.. In (B), parallel cultures from (A) were switched to DM for 48 h and lysates were made. Western analysis to detect MHC, with actin functioning as a loading and transfer control, was performed. For each shown are results for one experiment that are representative of three independent experiments.

(GM, which contains 10-15% serum) to differentiation media (DM, which contains <1% serum) for 48 hours. Western analysis for Myosin Heavy Chain (MHC), a biochemical marker for differentiation, was then performed (Fig 2B). MHC expression was detected in a dose-dependent fashion. At Myf5 expression levels comparable to the endogenous level detected in the C2C12 myoblasts (125ng pcMyf5 per 10^5 cells), MHC was detectable, confirming the ability of Myf5 to induce differentiation in fibroblasts.

The ectopic expression of MyoD has been shown by our laboratory and others to be capable of inducing differentiation in 10T ½ fibroblasts (Tapscott et al. 1988). In addition, our laboratory has discovered that MyoD expression is sufficient to induce apoptosis in fibroblasts when cultured in DM. Since Myf5's role in inducing differentiation seems to parallel that of MyoD, this raised the interesting question of whether its capability to induce apoptosis also follows.

To test this hypothesis, we transiently transfected 10T ½ fibroblasts with varying doses of pcMyf5 and assayed for ectopic Myf5 expression via Western analysis after overnight culture in GM (Fig 3A). Following this, parallel cultures were then switched to DM for six hours and apoptosis was quantified using the Cell Death Detection ELISA Plus Kit (Roche Applied Science), which uses a colorimetric assay to measure the concentration of cytosolic nucleosomes in a sample (Fig 3B). 23A2 skeletal myoblasts were included as a positive control

and the assay is plotted as fold increase from GM to DM. With increasing doses

of pcMyf5, up to six times the dose which was comparable

A　Myf5 →

β-actin →

pcDNA　250ng　500ng　1ug
**　　　pcMyf5 pcMyf5 pcMyf5**

B

Cytosolic Nucleosomes (Fold Increase GM to DM)
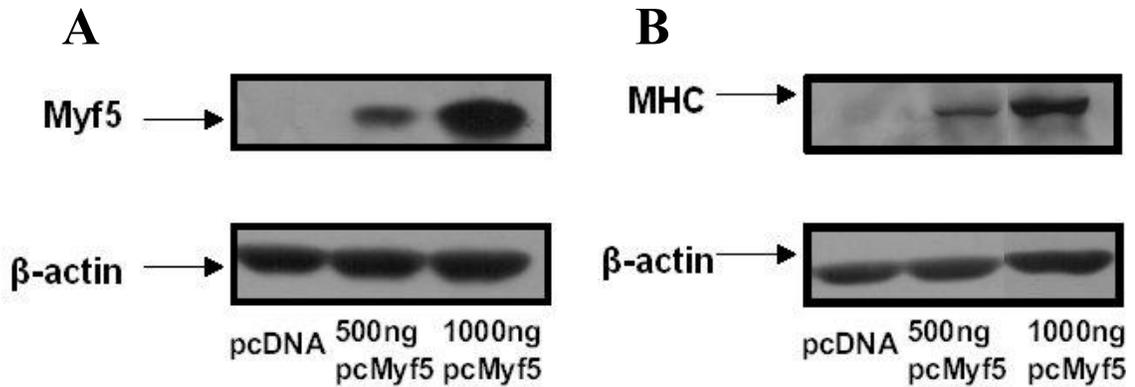
.03

.21　　　<.01　　<.01　　　<.01　　← **pcMyf5**

23A2

FIGURE 3. Ectopic Myf5 Expression Does Not Induce Apoptosis In Fibroblasts. In (A), equal cell numbers were plated and then transiently transfected with either pcDNA or pcMyf5 the next day. Following 24 h of culture in GM, lysates were made from the transfected fibroblasts. Western analysis for Myf5, with actin functioning as a loading and transfer control, was performed. In (B), parallel cultures from (A) were switched to DM for 6 h prior to the assessment of DNA fragmentation . DNA fragmentation was measured using the Cell Death Detection ELISA Plus Kit (Roche Applied Sciences) as described in Materials and Methods. For each shown are results for one experiment that are representative of three independent experiments. Standard error of the mean is given above each data point.

17

to endogenous levels of Myf5 in C2C12 myoblasts, we observed a modest to negligible increase in cytosolic nucleosomes (especially when compared to the 35-fold increase in the 23A2 myoblasts). These data indicate that ectopic Myf5, even when overexpressed, is insufficient to induce apoptosis in 10T½ fibroblasts. We then speculated that Myf5 might require an unknown protein, expressed in myoblasts but not fibroblasts, to induce apoptosis.

Our laboratory used RNAi to knock down MyoD levels in 23A2 skeletal myoblasts, creating A2:shMyoD cells. These A2:shMyoD cells were assayed for MyoD expression via Western analysis and cultured in DM for sufficient time to test for cytosolic nucleosomes (ELISA) or MHC, markers of apoptosis and differentiation, respectively. It was discovered that these MyoD silenced cells are defective for both differentiation and apoptosis (Terri Harford*,* personal observation*)*. Recalling that 23A2 myoblasts do not express detectable levels of Myf5 mRNA, we sought to find out whether ectopic Myf5 expression in these A2:shMyoD knockdowns would be sufficient to allow for differentiation and/or apoptosis. A2:shMyoD skeletal myoblasts were transiently transfected with either pcDNA3, 1ug pcMyf5 per 10^5 cells, or 2ug pcMyf5 per 10^5 cells. Following this, we detected Myf5 levels in GM via Western analysis (Fig. 4A) and quantified apoptosis in parallel cultures using the Cell Death Detection ELISA Plus Kit (Roche Applied Science) (Fig. 4B) following six hours of culture in DM. Increasing doses of Myf5 lead to a negligible increase in cytosolic nucleosomes.

A

Myf5 →

pcDNA 250ng 500ng 1000ng ← pcMyf5

β-actin →

B

Cytosolic Nucleosomes (Fold Increase GM to DM)

23A2   shMyoD   shMyoD   shMyoD   ← pcMyf5
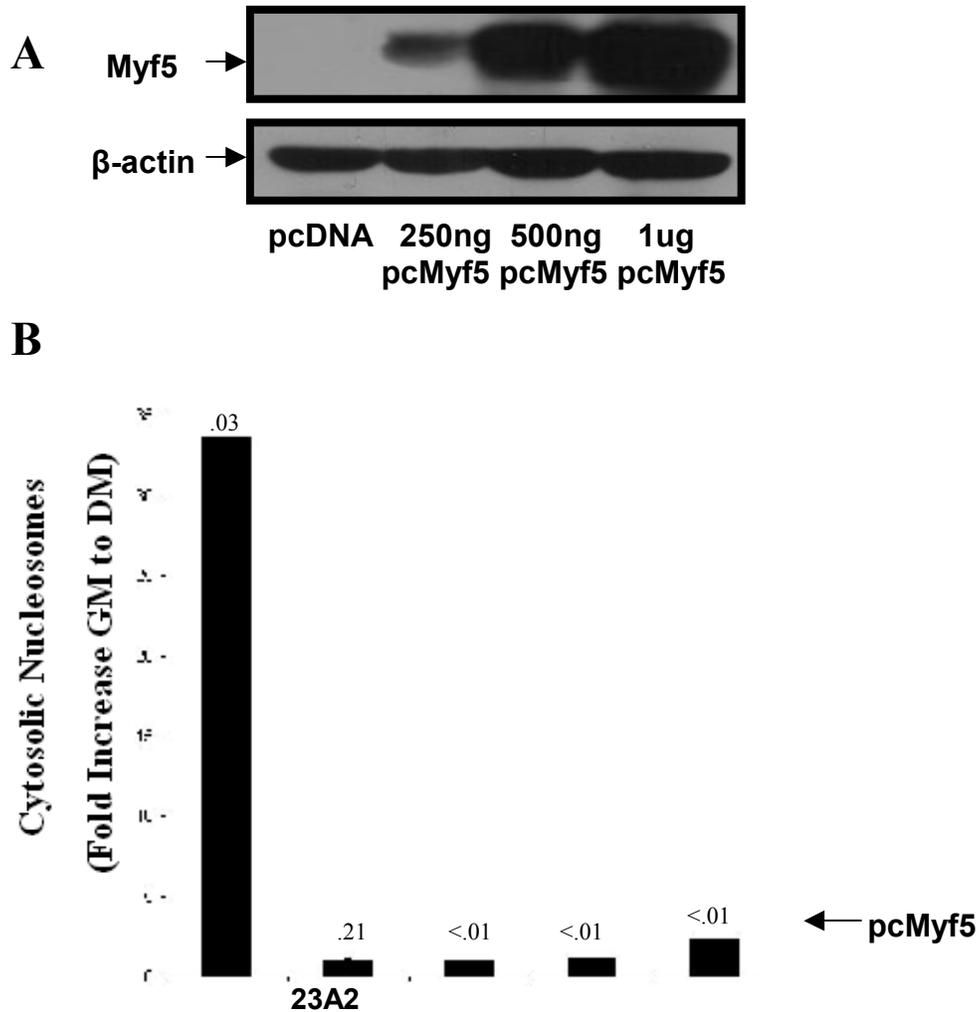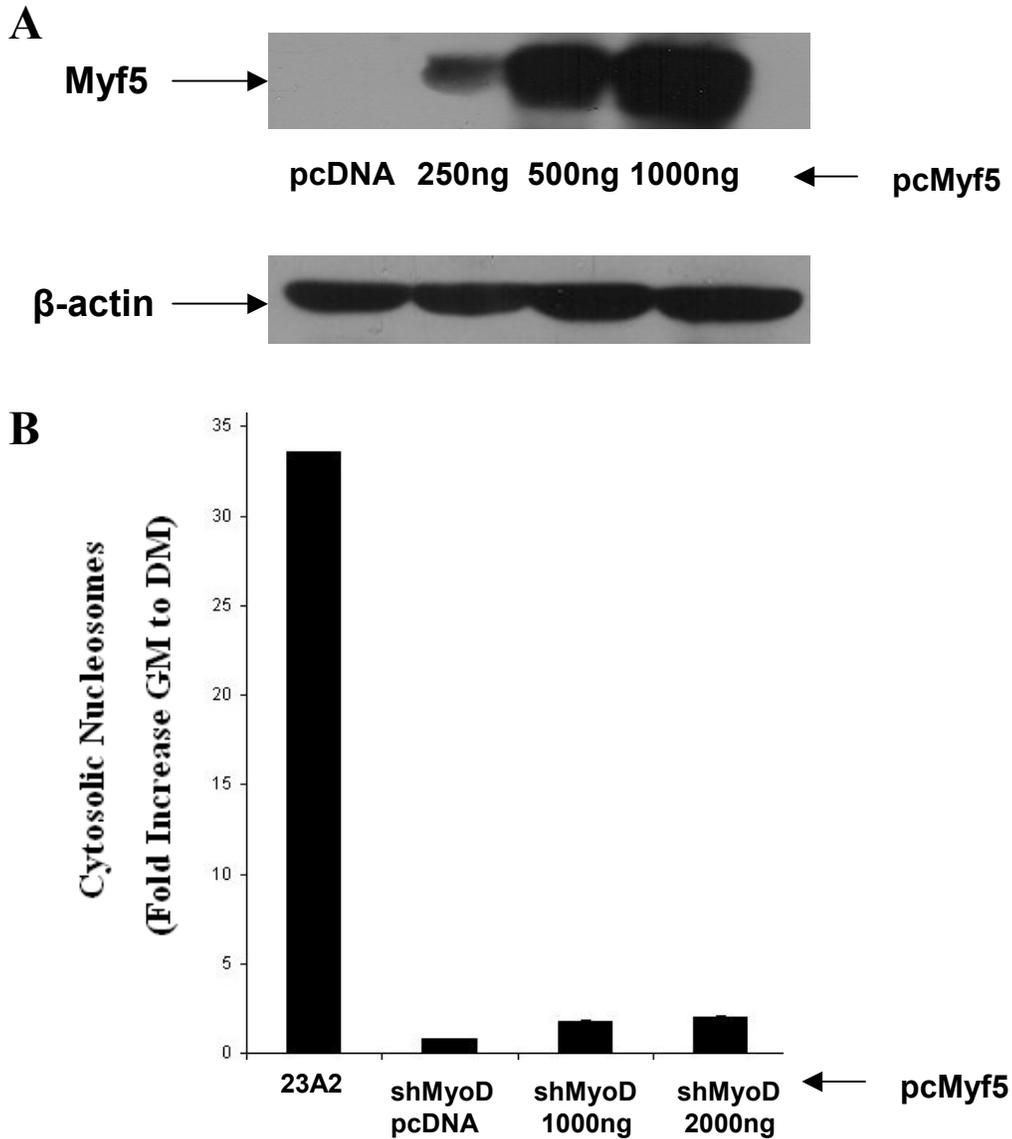   pcDNA   1000ng   2000ng

FIGURE 4. Ectopic Myf5 Expression Does Not Induce Apoptosis In A2:shMyoD Myoblasts. In (A), equal cell numbers were plated and then transiently transfected with either pcDNA or pcMyf5 the next day. Following 24 h of culture in GM, lysates were made from the transfected myoblasts. Western analysis for Myf5, with actin functioning as a loading and transfer control, was performed. In (B), parallel cultures from (A) were switched to DM for 7 h prior to the assessment of DNA fragmentation . DNA fragmentation was measured using the Cell Death Detection ELISA Plus Kit (Roche Applied Sciences) as described in Materials and Methods. For each shown are results for one experiment that are representative of two independent experiments. Standard error of the mean is given above each data point.

These data indicate that ectopic Myf5 expression was insufficient to restore apoptosis in both 10T½ fibroblasts and A2:shMyoD skeletal myoblasts.

3.2 Oncogenic Ras Inhibits the Expression Of Myf5 In Both Fibroblasts And Myoblasts.

Others have reported that MyoD expression is inhibited in skeletal myoblasts expressing oncogenic Ras. In order to determine whether oncogenic Ras also inhibits Myf5 expression, we planned to compare expression levels of ectopic Myf5 in 10T½ fibroblasts with expression levels of ectopic Myf5 in 10T½ fibroblasts expressing G12V H-Ras expressing fibroblasts. We also planned to perform a similar analysis in 23A2 and A2:G12V H-Ras myoblasts. However, we first needed to compare the transfection efficiency of these cell lines. To accomplish this, we compared expression of ectopically expressed β-galactosidase in transiently transfected 10T½ fibroblasts, 10T½:G12V H-Ras fibroblasts, 23A2 myoblasts, and A2:G12V H-Ras myoblasts (Fig. 5). We found similar levels of β-gal expression in the three cell lines tested. Next, we transiently transfected 10T½ and 10T½:G12V H-Ras fibroblasts with pcMyf5. After overnight culture in GM, we performed Western analysis to assess Myf5 levels in both cells lines and observed a reduction in expression in the 10T½:G12V H-Ras fibroblasts relative to the 10T½ fibroblasts (Fig 6).

We then tested for Myf5 expression by Western analysis after transiently transfecting 23A2 skeletal myoblasts and A2:G12V H-Ras myoblasts with increasing doses of pcMyf5 (Fig 7). A noticeable reduction in Myf5 expression occurred in the A2:G12V myoblasts relative to the 23A2 myoblasts. These data, when taken together, demonstrate that Myf5 expression is inhibited by the presence of oncogenic Ras in both skeletal myoblast and fibroblast lineages.
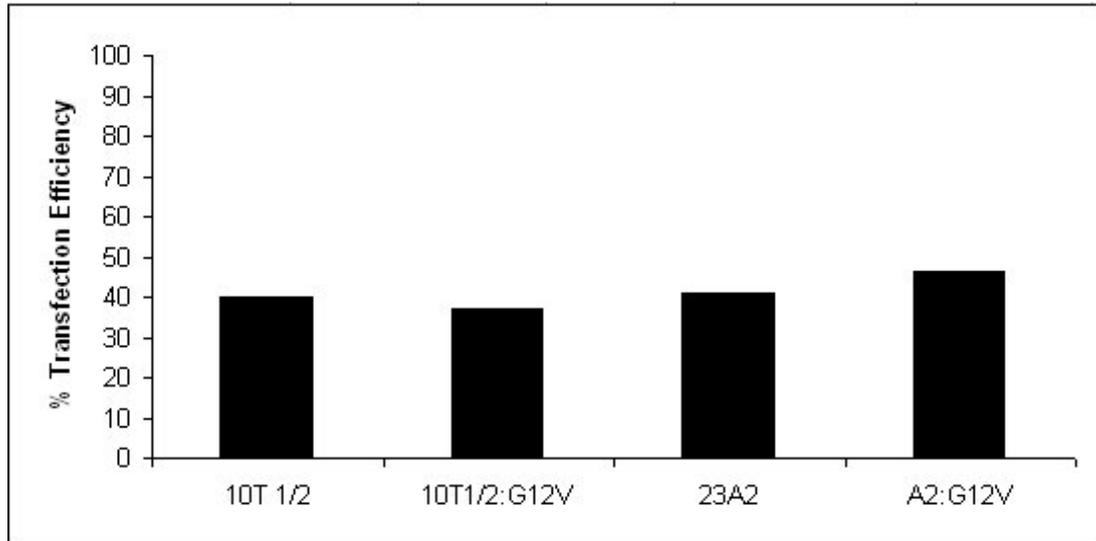
FIGURE 5. Transfection Efficiency In Four Fibroblast and Myoblast Cell Lines. Equal cell numbers were plated and then transiently transfected with B-gal the next day. After 24 hours of culture in GM, Cells were fixed and stained overnight with X-gal solution. The proportion of stained cells to total cells in a field of view was calculated and plotted as a percentage. Error bars represent the standard deviation of the mean from three independendent simultaneous experiments.

FIGURE 6. Ectopic Myf5 Expression Is Inhibited In 10T½:G12V H-Ras Fibroblasts. In (A), equal numbers of cells were plated and then transiently transfected with either pcDNA or pcMyf5 the next day. Following 24 h of culture in GM, lysates were made from the transfected fibroblasts. Western analysis for Myf5, with actin functioning as a loading and transfer control, was performed. For each shown are results for one experiment that are representative of three independent experiments. In (B), densitometry analysis was used to quantitate protein levels, normalizing to b-actin expression.
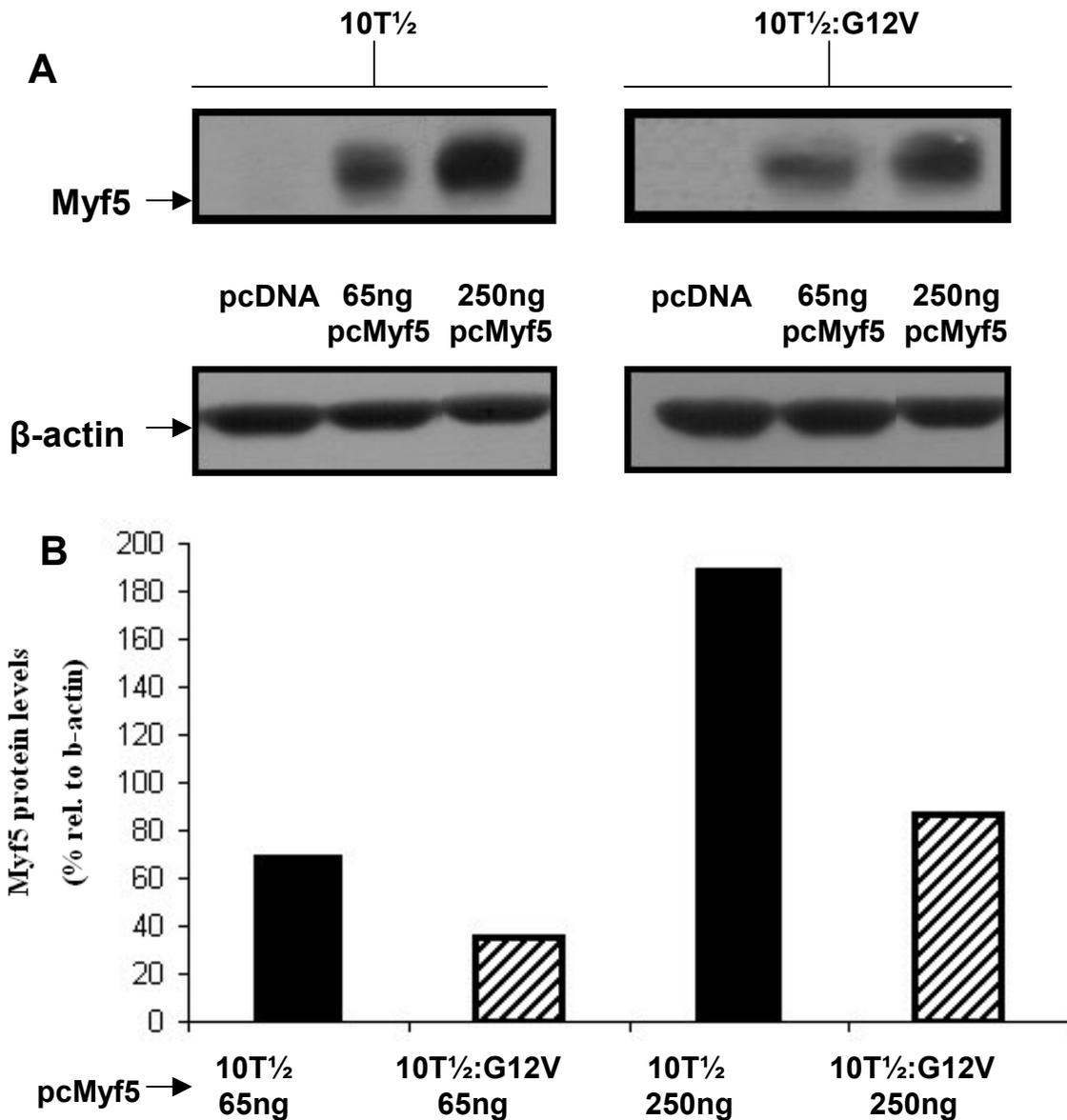
FIGURE 7. Ectopic Myf5 Expression Is Inhibited In A2:G12V H-Ras Myoblasts. In (A), equal numbers of cells were plated and then transiently transfected with either pcDNA or pcMyf5 the next day. Following 24 h of culture in GM, lysates were made from the transfected myoblasts. Western analysis for Myf5, with actin functioning as a loading and transfer control, was performed. For each shown are results for one experiment that are representative of two independent experiments. In (B), densitometry analysis was used to quantitate protein levels, normalizing to b-actin expression.
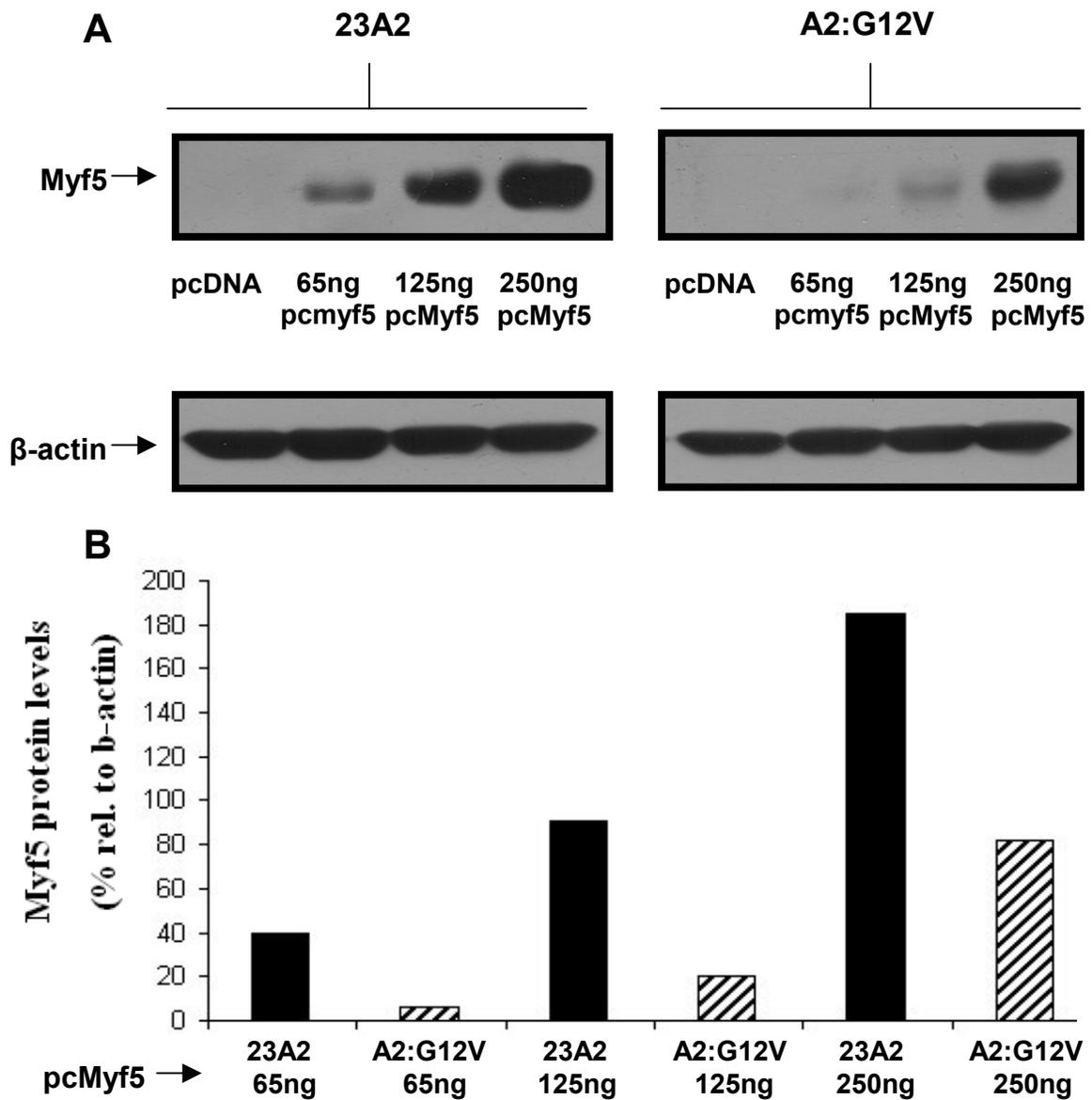
3.3 Assessment Of the Mechanism Of Oncogenic Ras Inhibition Of Ectopic Myf5 Expression

To uncover the mechanism of the inhibition of ectopic Myf5 expression by oncogenic Ras, we first sought to assess the levels of Myf5 mRNA. All four cell lines were transfected with equal amounts of pcMyf5 or pcDNA3. After overnight culture in GM, RNA was isolated from lysates and reverse-transcribed to cDNA. Myf5 levels were then detected by Realtime PCR. Similar levels of Myf5 mRNA were observed in each of the four cell lines (Fig 8).

We next assayed the effect of oncogenic Ras on the half-life of Myf5 protein. We transiently transfected 23A2 myoblasts and 23A2:G12V H-Ras myoblasts with 250ng pcMyf5 per 10^5 cells. Following overnight culture in GM, the plates of transfected 23A2 cells were pooled and the plates of transfected 23A2:G12V cells were pooled to control for transfection efficiency. These cells were then replated at $4x10^5$ cells per 100mm culture dish. Following overnight culture, cycloheximide was added to the plates to halt global translation. Lysates were made at various timepoints and assayed for Myf5 expression by Western Analysis (Fig 9) and the halflife of Myf5 was assessed by densitometry analysis. The presence of oncogenic Ras did not reduce the half-life of Myf5 protein.

Concurrently, the same experiment was performed in two fibroblast lineages, 10T½ and 10T½:G12V H-Ras (Fig 10). In this case, again, the presence of oncogenic Ras did not reduce the half-life of Myf5 protein.

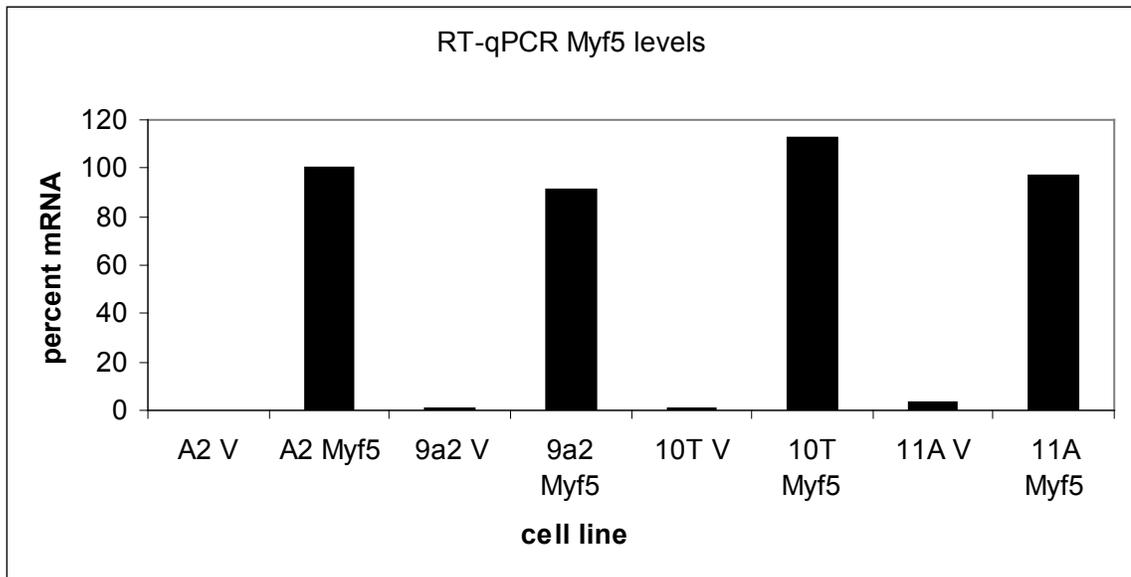**FIGURE 8. Oncogenic Ras Does Not Affect the mRNA Levels Of Ectopic Myf5.** Equal   numbers of cells were plated and then transiently transfected with 250ng pcMyf5 the next day. Following 24 h of culture in GM cells were lysed and RNA extraction was performed. Myf5 cDNA was transcribed by reverse transcription via SuperScript III (Invitrogen). Quantitative realtime PCR was performed and subsequent C(t) analysis is shown.

FIGURE 9. Oncogenic Ras Does Not Reduce the Half-life Of Myf5 Protein In Skeletal Myoblasts. Equal numbers of cells were plated and then transiently transfected with pcMyf5 the next day. Following 24 h of culture in GM, cells were pooled and replated at 4×10^5. Following overnight culture in GM, cells were treated with a timecourse of cycloheximide for 0, 30, 60, 120, or 180 minutes. Lysates were made. Western analysis for Myf5, with actin functioning as a loading and transfer control, was performed.
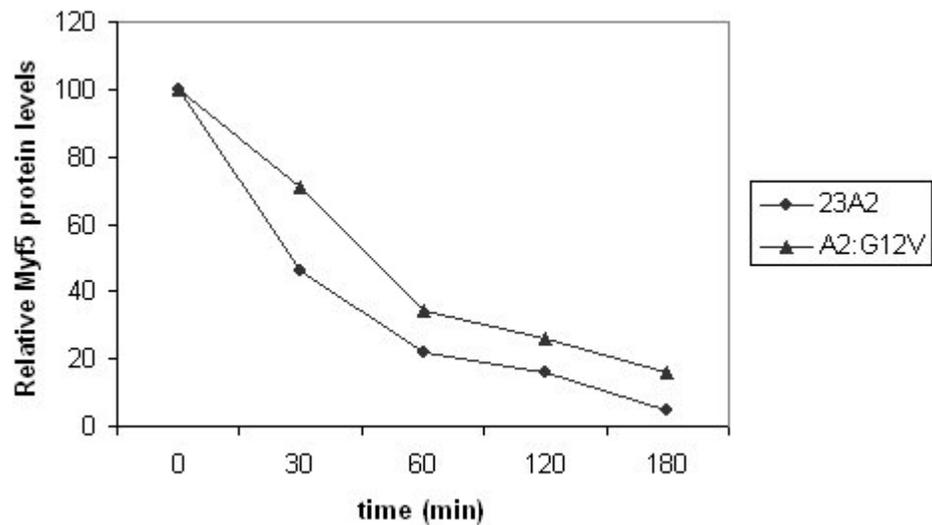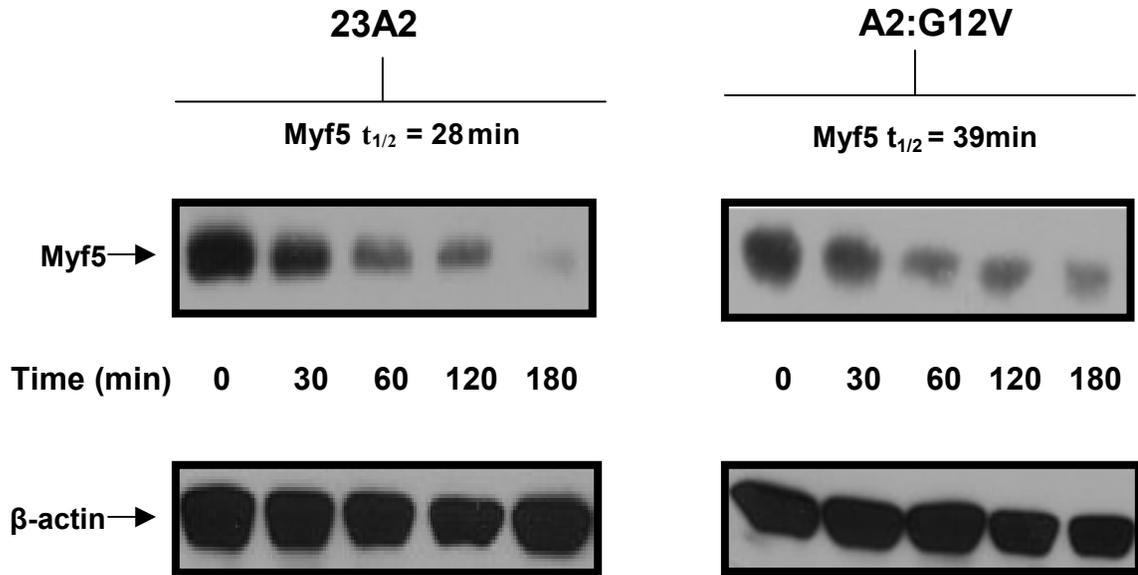
FIGURE 10. Oncogenic Ras Does Not Reduce the Half-life Of Myf5 Protein In Fibroblasts. Equal numbers of cells were plated and then transiently transfected with pcMyf5 the next day. Following 24 h of culture in GM, cells were pooled and replated at 4×10^5. Following overnight culture in GM, cells were treated with a timecourse of cycloheximide for 0, 30, 60, 120, or 180 minutes. Lysates were made. Western analysis for Myf5, with actin functionin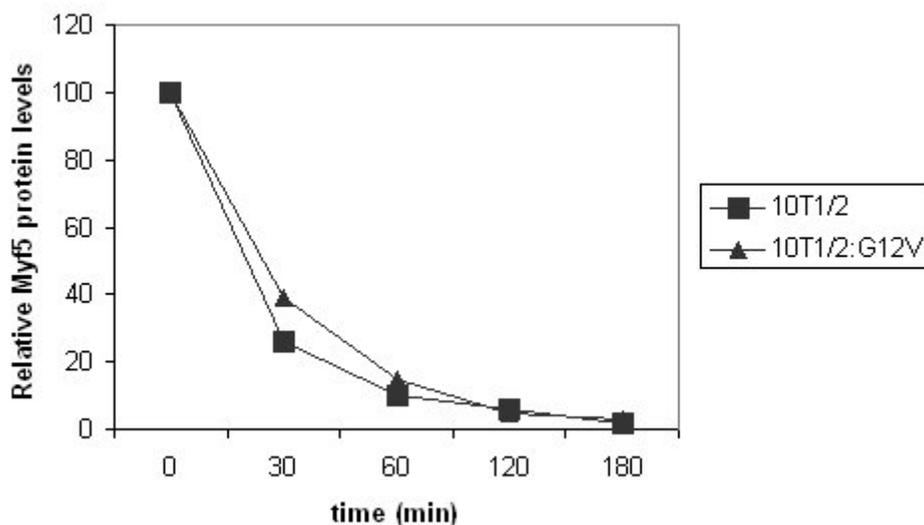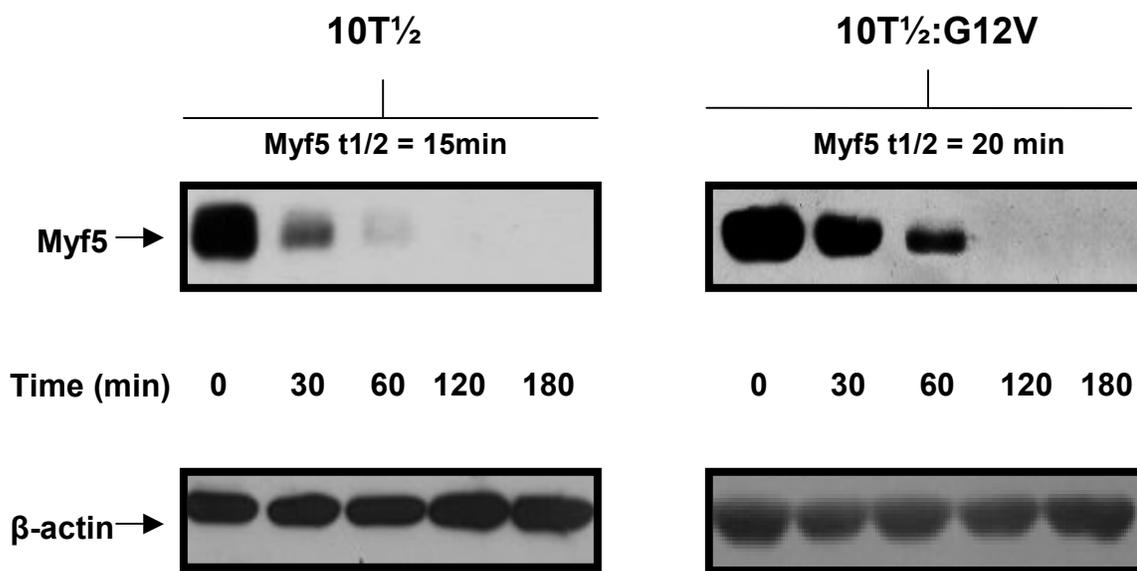g as a loading and transfer control, was performed. Densitometry analysis was performed to determine protein half-life. Sample bands were normalized to actin.

CHAPTER IV

DISCUSSION

4.1 Myf5 Plays a Role In Skeletal Myoblast Differentiation But Not the Coordinated Apoptosis.

While differentiation and apoptosis are well-established as critical processes for vertebrate development, knowledge of the coordinate regulation of these processes at the molecular level is not well understood. Skeletal myogenesis has long served as the paradigm in which to study the molecular regulation of differentiation since it is easily recapitulated using cell culture models. Culture in media lacking serum (differentiation media: DM) mimics the decreasing gradient of growth factors required *in vivo* to allow the degredation of Id, thus allowing MyoD and Myf5 to function. Using these cell culture models, our lab and others have reported that when cultured in DM, 30 percent of myoblasts undergo apoptosis rather than differentiation (Walsh 1997; Wang et al. 1997). Further, expression of oncogenic Ras blocks both differentiation and

apoptosis (Weyman 1997). Thus, differentiation and apoptosis are each induced by culture in DM, while each is inhibited by expression of oncogenic Ras. 10T½ fibroblasts quiesce when cultured in DM (Boraldi et al. 2008).

Both the MyoD and Myf5 transcription factors are capable of converting multipotential 10T½ fibroblasts into determined myoblasts that express muscle-specific genes when cultured in DM. Further, expression of MyoD in 10T ½ fibroblasts has recently been shown to be sufficient to allow for apoptosis in response to culture in DM (Terri Harford).

However, data presented herein has shown that expression of Myf5 is not sufficient to allow for apoptosis in 10T½ fibroblasts cultured in DM. Further, in 23A2 skeletal myoblasts that do not express endogenous Myf5 and that are silenced for MyoD expression as a consequence of RNAi, we have shown that ectopic expression of Myf5 cannot compensate for the ability of MyoD to allow for apoptosis.

Our findings are consistent with the work of others. Recently, these MyoD null myoblasts were shown to have increased survival following transplantation suggesting an impaired apoptotic phenotype. Myoblasts derived from MyoD null mice have a fivefold elevation in the expression of Myf5, yet display an impaired differentiation phenotype (Smythe and Grounds 2001). These results support a role for MyoD, but not Myf5, in both the differentiation and apoptotic processes

during regeneration. Finally, it is broadly accepted that Myf5 compensates for the absence of MyoD during developmental differentiation. However, if apoptosis is to play a key role during developmental myogenesis, we must speculate that a molecule other than MyoD can contribute to the apoptotic process since MyoD null mice are developmentally normal.

4.2 Myf5 Expression Is Regulated By Oncogenic Ras

Cancer is considered a disease of defective differentiation and defective apoptosis. Perhaps it should have been expected, then, that proteins capable of inducing cancer would regulate molecules responsible for inducing differentiation and/or apoptosis. Abrogation of MyoD expression by oncogenic Ras in skeletal myoblasts was reported almost 20 years ago (Olson et al. 1987). However, the mechanism for this inhibition has not yet been discovered. Likewise, here we have shown that the expression of oncogenic Ras is sufficient to diminish expression of Myf5 in fibroblasts and myoblasts. This leaves open the possibility that signaling through oncogenic Ras is affecting both MyoD and Myf5 through a common mechanism.

There are several different mechanisms by which oncogenic Ras could be affecting the expression of Myf5. One such model can be eliminated immediately. As the Myf5 expression we are dealing with is ectopic, it is driven by the strong CMV promoter located on the vector and should therefore overcome

any transcriptional inhibition present. This is not to say that oncogenic H-Ras does not downregulate the endogenous transcription of the *Myf5* gene. However, testing this hypothesis would require stable expression of *H-Ras* in a myoblast cell line which endogenously expressed Myf5 such as C2C12.

Oncogenic Ras could result in a decreased half-life of Myf5 mRNA. Our results showing equal levels of Myf5 mRNA regardless of the expression of oncogenic Ras indicate that this is not the case. However, rigorous proof would require the use of Northern hybridization to determine whether or not Ras affects Myf5's messenger RNA half-life. Once again, it is worth noting that this is the half-life of ectopic Myf5, and studying oncogenic Ras's effects on endogenous Myf5 would require a myoblast cell line which stably expresses it.

Oncogenic Ras could be downregulating the expression of Myf5 by affecting its protein stability. Our results show that in two different sets of experiments this does not appear to be the case (Figs. 9-10). In both fibroblasts and myoblasts, the protein half-life of Myf5 is not reduced; in point of fact, it is very slightly increased.

Finally, oncogenic Ras could affect the translation of Myf5. This could be effectively tested through S^{35}-methionine metabolic labeling followed by immunoprecipitation and quantitative comparison of the labeled Myf5 from parentals and cells expressing oncogenic Ras. Elucidating the precise

mechanism whereby oncogenic Ras prevents the expression of MyoD and/or

Myf5 might identify anti-cancer therapeutic targets.

LITERATURE CITED

Andres, V. and K. Walsh (1996). "Myogenin expression, cell cycle withdrawal, and phenotypic differentiation are temporally separable events that precede cell fusion upon myogenesis." J Cell Biol **132**(4): 657-66.

Arnold, H. H. and T. Braun (1996). "Targeted inactivation of myogenic factor genes reveals their role during mouse myogenesis: a review." Int J Dev Biol **40**(1): 345-53.

Bodine-Fowler, S. (1994). "Skeletal muscle regeneration after injury: an overview." J Voice **8**(1): 53-62.

Boraldi, F., G. Annovi, et al. (2008). "The effect of serum withdrawal on the protein profile of quiescent human dermal fibroblasts in primary cell culture." Proteomics **8**(1): 66-82.

Chang, T. H., M. Primig, et al. (2004). "An enhancer directs differential expression of the linked Mrf4 and Myf5 myogenic regulatory genes in the mouse." Dev Biol **269**(2): 595-608.

Chang, T. H., S. D. Vincent, et al. (2007). "The A17 enhancer directs expression of Myf5 to muscle satellite cells but Mrf4 to myonuclei." Dev Dyn **236**(12): 3419-26.

Emerson, C. P., Jr. (1993). "Embryonic signals for skeletal myogenesis: arriving at the beginning." Curr Opin Cell Biol **5**(6): 1057-64.

Gayraud-Morel, B., F. Chretien, et al. (2007). "A role for the myogenic determination gene Myf5 in adult regenerative myogenesis." Dev Biol **312**(1): 13-28.

Kassar-Duchossoy, L., B. Gayraud-Morel, et al. (2004). "Mrf4 determines skeletal muscle identity in Myf5:Myod double-mutant mice." Nature **431**(7007): 466-71.

Konieczny, S. F., B. L. Drobes, et al. (1989). "Inhibition of myogenic differentiation by the H-ras oncogene is associated with the down regulation of the MyoD1 gene." Oncogene **4**(4): 473-81.

Konigsberg, I. R. (1971). "Diffusion-mediated control of myoblast fusion." Dev Biol **26**(1): 133-52.

Lathrop, B., E. Olson, et al. (1985). "Control by fibroblast growth factor of differentiation in the BC3H1 muscle cell line." <u>J Cell Biol</u> **100**(5): 1540-7.

Linardic, C. M. and C. M. Counter (2008). "Genetic modeling of Ras-induced human rhabdomyosarcoma." <u>Methods Enzymol</u> **438**: 419-27.

Mal, A., M. Sturniolo, et al. (2001). "A role for histone deacetylase HDAC1 in modulating the transcriptional activity of MyoD: inhibition of the myogenic program." <u>Embo J</u> **20**(7): 1739-53.

Mauro, A. (1961). "Satellite cell of skeletal muscle fibers." <u>J Biophys Biochem Cytol</u> **9**: 493-5.

Miller, J. B. and M. Girgenrath (2006). "The role of apoptosis in neuromuscular diseases and prospects for anti-apoptosis therapy." <u>Trends Mol Med</u> **12**(6): 279-86.

Montarras, D., C. Lindon, et al. (2000). "Cultured myf5 null and myoD null muscle precursor cells display distinct growth defects." <u>Biol Cell</u> **92**(8-9): 565-72.

Olson, E. N. (1992). "Interplay between proliferation and differentiation within the myogenic lineage." <u>Dev Biol</u> **154**(2): 261-72.

Olson, E. N., G. Spizz, et al. (1987). "The oncogenic forms of N-ras or H-ras prevent skeletal myoblast differentiation." <u>Mol Cell Biol</u> **7**(6): 2104-11.

Rudnicki, M. A. and R. Jaenisch (1995). "The MyoD family of transcription factors and skeletal myogenesis." <u>Bioessays</u> **17**(3): 203-9.

Scholl, F. A., D. R. Betts, et al. (2000). "Molecular features of a human rhabdomyosarcoma cell line with spontaneous metastatic progression." <u>Br J Cancer</u> **82**(6): 1239-45.

Schultz, E. and K. M. McCormick (1994). "Skeletal muscle satellite cells." <u>Rev Physiol Biochem Pharmacol</u> **123**: 213-57.

Shen, X., J. M. Collier, et al. (2003). "Genome-wide examination of myoblast cell cycle withdrawal during differentiation." <u>Dev Dyn</u> **226**(1): 128-38.

Smythe, G. M. and M. D. Grounds (2001). "Absence of MyoD increases donor myoblast migration into host muscle." <u>Exp Cell Res</u> **267**(2): 267-74.

Spandidos, D. A. (1986). "A unified theory for the development of cancer." <u>Biosci Rep</u> **6**(8): 691-708.

Tapscott, S. J. (2005). "The circuitry of a master switch: Myod and the regulation of skeletal muscle gene transcription." Development **132**(12): 2685-95.

Tapscott, S. J., R. L. Davis, et al. (1988). "MyoD1: a nuclear phosphoprotein requiring a Myc homology region to convert fibroblasts to myoblasts." Science **242**(4877): 405-11.

Ustanina, S., J. Carvajal, et al. (2007). "The myogenic factor Myf5 supports efficient skeletal muscle regeneration by enabling transient myoblast amplification." Stem Cells **25**(8): 2006-16.

Walsh, K. (1997). "Coordinate regulation of cell cycle and apoptosis during myogenesis." Prog Cell Cycle Res **3**: 53-8.

Wang, J., K. Guo, et al. (1997). "Rb functions to inhibit apoptosis during myocyte differentiation." Cancer Res **57**(3): 351-4.

Wang, J. and K. Walsh (1996). "Resistance to apoptosis conferred by Cdk inhibitors during myocyte differentiation." Science **273**(5273): 359-61.

Weyman, C. M. and A. Wolfman (1997). "Oncogenic Ras-induced secretion of a novel inhibitor of skeletal myoblast differentiation." Oncogene **15**(21): 2521-8.

Weyman, C.M. et al. (2002). "Apoptosis coincident with the differentiation of skeletal myoblasts is delayed by caspase 3 inhibition and abrogated by MEK-independent constitutive Ras signaling." Cell Death Differ **9**(2): 209-18.

Yablonka-Reuveni, Z., K. Day, et al. (2008). "Defining the transcriptional signature of skeletal muscle stem cells." J Anim Sci **86**(14 Suppl): E207-16.

Yablonka-Reuveni, Z., M. A. Rudnicki, et al. (1999). "The transition from proliferation to differentiation is delayed in satellite cells from mice lacking MyoD." Dev Biol **210**(2): 440-55.