Amplification and Overexpression of the MDM2 Oncogene Inhibits Muscle Differentiation in Rhabdomyosarcoma

By

TROY ALLEN FIDDLER

A

DISSERTATION

Presented to the Department of Molecular and Medical Genetics

and the Oregon Health Sciences University

School of Medicine

in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

September 1997

Approval Sheet

School of Medicine
Oregon Health Sciences University

CERTIFICATE OF APPROVAL

This is certification that the Ph.D. dissertation of

TROY ALLEN FIDDLER

has been approved

[redacted]

Professor in charge of dissertation

Member [redacted]

Memb[redacted]

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M[redacted]

Member

Associate Dean for Graduate Studies

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Acknowledgements

I would like to thank my graduate advisor and friend, Dr. Matt Thayer for his support and assistance throughout my graduate work. I would also express my condolences for all those lost 49er games, which proved beneficial to myself, yet detrimental to my liver.

I would like to express my thankfulness to the other members of my graduate advisory committee; Dr. Markus Grompe, Dr. Michael Liskay, Dr. Stan Hollenberg, and Dr. William Horton for their helpful advice and guidance throughout graduate school.

I want to thank all the members in the Thayer laboratory, Terasa Johnson-Pais, Catherine Degnin, Shujing Liu and especially Leslie Smith for their friendship and support. I would also like to thank fellow graduate students Paul Rupp and Felix Munoz for their friendship and for hours of computerized mayhem, destruction, and anarchy.

I would like to convey my gratefulness to my wife and friend Elizabeth Odell-Fiddler for her friendship and support, as well as, for a critical evaluation of this manuscript. I would also like to express gratitude to my son Connor (the Master) Fiddler for his assistance in editing the manuscript and slide presentation, as well as, his support for my interest in computer games.

I would like to thank all those members of our soccer team, FC77 Saxer, for two championship trophies, and for all those timely substitutions when my serum testosterone levels became elevated.

I would express my indebtedness to my Father (Gary Fiddler) and my Mother (Fran Fiddler) for their support and understanding over past thirty years.

I would like to dedicate this work to Dr. Dennis Hart, who helped open the eyes of a troubled teenager, to the beauty of Science and the scientific process. This manuscript would not exist today if not for your guidance, trust, and friendship. Thank you.

Abstract

Rhabdomyosarcomas are one of the most common solid tumors of childhood, representing 4-8% of all malignant diseases under 15 years of age. Tumors arise de novo from skeletal muscle, and in some instances demonstrate morphological characteristics of differentiated muscle tissue. Sarcomas have traditionally been classified as rhabdomyosarcomas based on morphology and expression of muscle structural genes, such as myosin heavy chain or desmin. Expression of the muscle determination gene MyoD has been shown to be the most sensitive marker for classifying sarcomas as rhabdomyosarcomas. Although expression of the MyoD family has been demonstrated in rhabdomyosarcoma, it appears that these potent muscle program activators lack the ability to orchestrate terminal differentiation in these tumors. We have classified various rhabdomyosarcoma cells lines as having either a recessive or dominant non-differentiating phenotype. This study is an analysis on the overt failure to differentiate in the rhabdomyosarcoma classified as dominant non-differentiating rhabdomyosarcoma.

To study the genetic basis of the dominant non-muscle phenotype we transferred chromosomes from rhabdomyosarcoma cell into C2C12 mouse myoblasts. Transfer of a single derivative 14 chromosome inhibited muscle differentiation. Gene amplification and chromosomal rearrangements are associated with many tumor types. These genetic abnormalities often result in aberrant gene expression. We characterized the derivative 14 chromosome with regard to the presence or absence of a variety of genomic and cDNA markers. MDM2 was found to be amplified and over in the non-differentiating hybrids and the parental rhabdomyosarcoma. Furthermore, forced expression of MDM2 was

shown to inhibit MyoD and myf-5 dependent gene transcription. While, constitutive MDM2 expression repressed muscle-specific gene transcription, forced expression of antisense MDM2 resulted in rescued muscle-specific gene transcription.

Analysis on the inhibition of MyoD function by MDM2 revealed that loss of the MyoD protein was not required for MDM2 repression of MyoD-dependent transcription. Rather, MDM2 inhibition of muscle gene transcription was shown to be DNA site specific, requiring the presence of MRF binding sites. Expression of the MyoD family's synergistic partner, MEF-2C, rescued the non-muscle phenotype associated with overexpression of MDM2. In addition, we found that MDM2 repression to be independent of p53. Contrary to p53 and p21, expression of pRb alleviated the MDM2 inhibition of muscle-specific gene transcription. We therefore conclude that amplification and overexpression of MDM2 inhibits MRF medicated muscle gene transcription through two potential mechanisms: 1) inhibition of MEF-2C activity, and 2) inhibition of pRb function.

Introduction

The MyoD – Family and Myogenesis

In vertebrates skeletal muscle is derived from the somites, which are segmented units of paraxial mesoderm formed on either side of the neural tube (Buckingham, 1994; Rong et al., 1992). Under the influence of various extracelluar stimuli, multi-potent cells from within the paraxial mesoderm differentiate into the dorsal dermomyotome and the ventral sclerotome. The sclerotome will eventually give rise to cartilage and bone of the vertebral column and ribs (Yun and Wold, 1996). Cells constituting the dermomyotome are the progenitors of muscle tissue. Specifically, populations of cells contributing to the dorsal-medial region in the somite migrate from the dermomyotome to from the myotome. The myotome consists of muscle progenitor cells with the ability to differentiate into post-mitotic differentiated skeletal muscle. The progenitor cells that become committed to muscle differentiation are termed myoblasts. Myoblasts have the ability to proliferate and migrate, and following the proper sequence of environmental cues begin to express determination – class muscle regulatory factors (MRFs), exit the cell cycle, and differentiate into mature muscle cells termed myocytes. Following withdrawal from the cell cycle myocytes undergo cellular fusion to form multi-nucleated syncytia which, following organized arrangement, form muscle fibers (Figure Intro-1).

The MyoD family of muscle regulatory factors includes, MyoD, myf-5, myogenin, and MRF4, and constitutes a subset of basic Helix-Loop-Helix (bHLH) transcription factors. There are at least three classes of HLH proteins, which are defined by their dimerization preferences. Class A bHLH proteins constitutes those HLH proteins that are present in a wide variety of cell types, and preferentially dimerize with class B

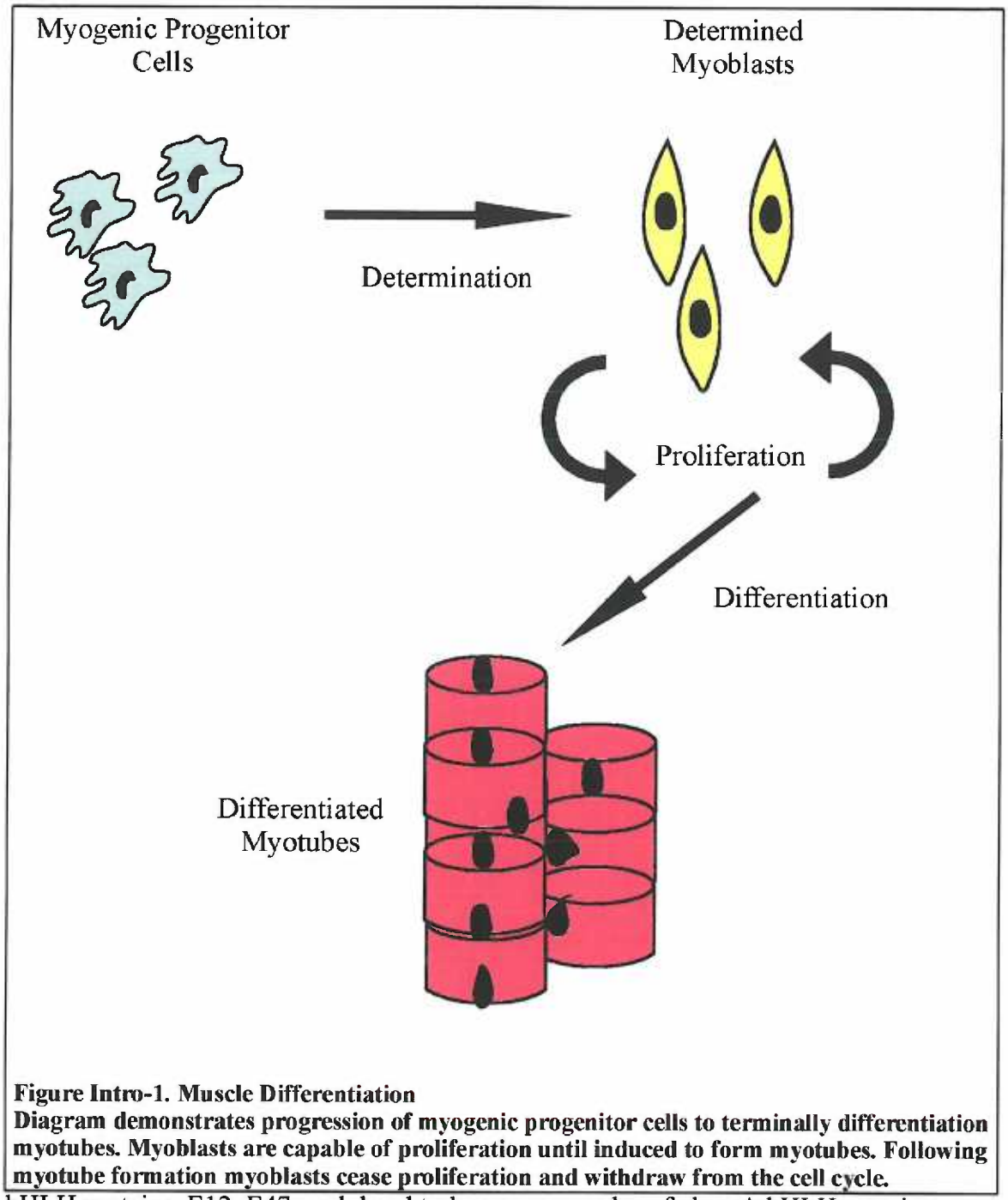

Figure Intro-1. Muscle Differentiation
Diagram demonstrates progression of myogenic progenitor cells to terminally differentiation myotubes. Myoblasts are capable of proliferation until induced to form myotubes. Following myotube formation myoblasts cease proliferation and withdraw from the cell cycle.

bHLH proteins. E12, E47, and daughterless are examples of class A bHLH proteins. Class B bHLH proteins, such as MyoD, myogenin, and achaete-scute tend to be tissue specific in distribution and favor class A bHLH molecules as dimerization partners. The class C proteins include the products of the myc family, which do not dimerize with either class A or class B bHLH proteins.

Though limb, head, and trunk skeletal muscles develop as separate lineages in vertebrates, all rely on members of the same molecular network to regulate their differentiation (Smith et al., 1994). Myf-5 is the first myogenic regulatory gene to be expressed during mouse embryogenesis, with transcripts appearing in the somite at day 8.0-postcoitum (p.c.). Myogenin transcripts appear in the myotome beginning at day 8.5 p.c., and MRF4 and MyoD are expressed in the myotome beginning at day 9.0 p.c. and 10.5 p.c., respectively (Patapountian et al., 1993). In the limb buds, myf-5 transcripts appear at day 11.0 p.c., with myogenin and MyoD mRNA expressed about twelve hours later (Ott et al., 1991; Patapountian et al., 1993). MRF4 transcripts do not accumulate in the muscle fibers of the limbs until birth. After birth MRF4 becomes up-regulated and is the predominant member of the MyoD family to be expressed (Molkentin and Olson, 1996). While myoblasts destined to form limb musculature express MyoD at their first stage in muscle determination (Smith et al., 1994; Smith et al., 1993), additional studies indicate that cells committed to the formation of head and neck muscles initiate the muscle program by expression of myf-5. The altering expression patterns between the various muscle lineages raises the possibility that these proteins may function interchangeably. Differentiation in all three lineages is initiated by expression of myogenin and members of the myocyte enhancer factor 2 (MEF-2) family (Edmondson et al., 1994).

One of the most striking properties of vertebrate MRFs is that ectopic expression of any one of the four genes in the MyoD family, in a variety of cell lines, results in phenotypic conversion from a previous cell type into that of skeletal muscle (Braun et al., 1990; Braun et al., 1989; Davis et al., 1987; Edmondson and Olson, 1989). Forced

muscle differentiation includes expression of a wide array of muscle specific genes and ,in some instances, myotube formation. Contrary to data gained from tissue culture experiments, gene disruption studies suggest that no single MRF is absolutely required to command muscle differentiation in the developing mouse. It appears that skeletal muscle formation in the mouse requires coordinated activation of a muscle regulatory network, which is not confined to the MyoD family. Subsequent gene disruption studies on MRFs indicated that some type of cross-regulation must take place between the MRF family in the embryo. The different MRF genes were found to serve distinct roles in muscle development. The myf-5 and MyoD single knockouts resulted in the production of normal myocytes and muscle formation (Braun et al., 1992; Rudnicki et al., 1992). The Myf-5/MyoD double knockout failed to produce myoblasts and was lethal (Rudnicki et al., 1993). Thus myf-5 and MyoD have some degree of functional redundancy in establishing the commitment of progenitor cells to the myogenic program. In contrast, myogenin null mice are essentially void of mature myocytes, although they do appear to possess a large pool of myoblasts (Hasty et al., 1993; Nabeshima et al., 1993). Myogenin, therefore, functions to direct differentiation of myoblasts into mature myocytes.

MyoD Regulation of Gene Transcription

Regulation of target gene transcription by the bHLH proteins requires both protein-protein interaction as well as protein-DNA binding (Blackwell, 1990; Lassar et al., 1989; Molkentin et al., 1996; Murre et al., 1989). Members of the MyoD family share extensive amino acid homology within a basic region and the adjacent helix-loop-helix domain. Both motifs mediate distinct functions. The basic region of the protein is

involved with binding to target DNA sequences (Davis, 1992; Ma, 1994), while the helix-loop-helix domain engages in protein-protein interactions. All bHLH proteins that bind DNA recognize the consensus sequence CANNTG, which is referred to as the E-box (Blackwell, 1990; Lassar et al., 1989; Murre et al., 1989). Although most bHLH proteins bind the E-box, individual bHLH proteins display distinct half-site preferences for binding that depend on nucleotides within and surrounding the CANNTG consensus (Blackwell, 1990). Binding of the myogenic bHLH protein to DNA is enhanced by the presence of ubiquitously expressed E2A gene products (E12 and E47) (Bain, 1993). The MyoD family members preferentially form heterodimers with E-proteins (E12 or E47). Furthermore, the MyoD/E-protein heterodimers must be formed, in order to effectively bind DNA and initiate gene transcription (Blackwell, 1990).

E proteins also form heterodimers with other tissue specific bHLH proteins to regulate different sets of genes in tissues, such as, erythrocytes (Aplan, 1992), pancreas (Nelson et al., 1990), lymphocytes (Benerzra, 1994), and neurons (Jan, 1993). The ability of these different protein complexes to regulate different sets of genes appears paradoxical, since their DNA binding specificities are often overlapping. It therefore appears that additional protein factors are involved in determining transcriptional specificity. Recent experiments suggest that MyoD may require a positive cofactor to activate transcription of appropriate genes, and that transcription can be prevented at inappropriate sites by repressors that recognize bases overlapping the E-box (Tapscott et al., 1993; Thayer and Weintraub, 1990; Thayer and Weintraub, 1993; Weintraub et al., 1991). It appears that the function of the proposed cofactor and repressor both depend on the presence of MyoD basic region residues alanine114 and threonine115. Mutations

involving these sites, which result in the amino acid sequence identical to the E protein basic region, allow for DNA binding but not transcriptional activity (Davis, 1992). X-ray crystallography studies indicate that in bound complexes these residues are in the proximity of the major groove of the DNA (Ma, 1994). It appears that the MyoD basic region can assume a conformation different than that of the E protein basic region and that the conformational divergence by MyoD can allow important interactions between MyoD and both activating cofactors and repressors.

In addition to activating structural and functional genes associated with myogenesis, each member of the MyoD family can auto-regulate its own and cross-activate one another's' expression in transfected 10T1/2 cells (Thayer et al., 1989; Thayer and Weintraub, 1990; Weintraub et al., 1989; Yutzey et al., 1990). Such interactions have made it difficult to assign specific functions to the individuals of the myogenic family. Since the knockout data indicates that full functional redundancy within the MyoD family does not exist, it is likely that auto-regulation of the myogenic family may be a mechanism which provides stability to the muscle phenotype *via* amplification of multiple myogenic signals.

The myogenic regulators MyoD, myogenin, Myf-5, and MRF4 share approximately 80% amino acid identity within the basic helix-loop-helix region, which mediates protein dimerization and DNA binding (Atchley et al., 1994). In addition, transcriptional activation domains, which show limited sequence similarities, are located within the amino and carboxyl termini (Lassar et al., 1989; Weintraub et al., 1991). These activation domains are not essential for myogenic conversion of 10T1/2 cells. It appears that the E-protein dimerization partners also possess transcriptional activator domains,

which can function in the absence of a myogenic transcriptional activator region (Lassar et al., 1991). This suggests that the activator domains function as adapters to the transcriptional machinery. Mutational analysis of the MyoD protein revealed that increasing the size of a C-terminal deletion resulted in substantial increases in the mutant proteins' ability to act as a transcriptional activator (Davis, 1992; Weintraub et al., 1991). So it appears that changes in protein conformation result in enhanced transcriptional activity. Such conformational changes may occur through either the act of dimerization or by affecting binding affinity to target DNA sequences.

To determine if the MyoD transcriptional activator domain interacts with specific factors in the transcriptional machinery, domain swapping experiments were performed. The MyoD basic region was replaced with the basic region from the E12 protein termed MyoD-E12Basic. The resulting protein failed to activate the MCK enhancer or 4RtkCAT constructs in 10T1/2 and 3T3 cells (Davis, 1992; Weintraub et al., 1991). Furthermore, fusion of the VP16 activation domain to MyoD-E12Basic construct resulted in activation of 4Rtk-CAT at comparable levels with MyoD or MyoD-VP16 with both 10T1/2 and 3T3 cells (Weintraub et al., 1991). Therefore, although the MyoD activation domain functions only within the context of the correct basic region, the VP16 activation region can function independently of the activity governed by the basic region. VP16 could essentially be functioning as a universal mediator to the transcription machinery and transcriptional activation by MyoD may require interaction with a set of specific developmentally expressed cofactors to activate transcription.

Although MyoD-E12Basic failed to activate the 4RtkCAT construct in 10T1/2 or 3T3 cells, it was shown to activate the same reporters in both CV1 cells and B78

melanoma cells (Weintraub et al., 1991). These results emphasize the fact that MyoD-E12Basic can bind to the MyoD binding sights *in vivo*. The fact that activation occurred after DNA binding in the CV1 and B78 cells, but not 10T1/2 or 3T3 cells, suggests that a factor is available in CV1 and B78 cells, but not in 10T1/2 or 3T3 cells, that can recognize the E12Basic region in association with MyoD DNA binding sites.

E-boxes are present in the control regions of most muscle-specific genes and are important for muscle specific transcription (Jaynes et al., 1988; Moss et al., 1988; Nelson et al., 1990; Piette et al., 1990). There exist, however, muscle-specific genes that lack the presence of an E-box within their regulatory regions. Characterization of these regulatory regions identified AT rich sequences of DNA that complexed with the MEF-2 family of proteins (Takada et al., 1995). MEF-2 was originally identified as a muscle-specific DNA binding protein who's activity was induced when skeletal myoblasts differentiated into myotubes (Gosset et al., 1989). The MEF-2 family of transcription factors comprises a group of transcriptional activators, MEF-2A, MEF-2B, MEF-2C and MEF-2D, that show homology in a MADS (MCM1, Agamous, Deficiens, and Serum response factor) box and a proximal motif known as the MEF-2 domain (Shore and Sharrocks, 1995).

MEF2-C Synergizes with the MyoD Family

The MEF-2 family of proteins is capable of binding as homodimers and heterodimers to a consensus site C/TTA $(A/T)_4$ TAG/A (Olson et al., 1995). This consensus site has been found in the control regions of numerous muscle specific genes, as well as control regions governing expression of myogenic bHLH genes (Jaynes et al., 1988). Expression of the MEF-2 genes occurs early in the embryogenesis of myogenic

lineages. With transcripts appearing in the precardiac mesoderm at day 7.5 postcoitum (pc)(Edmondson et al., 1994), MEF-2C is the first member of the family to be expressed in the mouse. MEF-2C is expressed in the skeletal muscle myotome beginning at day 9.0 pc and is soon followed by expression of the other members of the family. Following birth, MEF-2C expression is restricted to skeletal muscle (Martin et al., 1993), brain, and spleen (McDermott et al., 1993), whereas the MEF-2A, MEF-2B, and MEF-2D are expressed ubiquitously (McDermott et al., 1993; Pollock and Treisman, 1991).

Unlike the myogenic bHLH factors, the MEF-2 family lack myogenic activity on their own, but have been shown to synergize with the myogenic bHLH factors to activate muscle gene expression (Molkentin et al., 1995). This synergy has been shown to be mediated by direct protein- protein interactions between MEF-2 factors and heterodimers formed between members of the MyoD family and E proteins. Co-immunoprecipitation and trihybrid assays demonstrated that the MADS/MEF2 domains of MEF2-C specifically interacted with the heterodimer formed between the bHLH regions of myogenin and E12 (Molkentin et al., 1995).

Myogenin deletion mutants containing only the bHLH region were unable to activate myogenesis in 10T1/2 fibroblasts, although this mutant was able to dimerize with E proteins and bind DNA (Molkentin et al., 1996). In the presence of wild type MEF-2C, the myogenin mutant induced myogenesis in 10T1/2 cells. This demonstrated that not only did the bHLH region of myogenin mediate the synergism between myogenin and MEF-2C, but that MEF-2C can act as a transcriptional activator for myogenic bHLH proteins. To define the MEF-2c domains which were required for synergism with myogenic bHLH factors, experiments were designed using wild type myogenin and

MyoD with various MEF-2C deletion mutants. The results of these experiments suggest that only the first 117 amino acids, which contain both the MADS box and MEF2 domain, of MEF-2C are required for synergism with either myogenin or MyoD (Molkentin et al., 1996). Together these results demonstrate that MEF-2C is a cofactor for myogenic bHLH proteins and that only a single transcriptional domain is required in either MEF-2C or myogenic bHLH proteins to activate endogenous muscle specific genes in 10T1/2 fibroblasts.

Extensive mutational analysis of the MEF-2C N-terminus was carried out to determine the regions of MEF-2C which are required for synergism with myogenic bHLH proteins (Molkentin et al., 1996). While still allowing for synergism with MyoD and myogenin in myogenic induction, mutations within the MADS domain abolished the ability of MEF-2C to bind DNA. Alternatively, mutations within the MADS domain which inhibited dimerization and mutations within the MEF2 region failed to synergize with myogenic bHLH proteins. This suggests that both the MADS and MEF2 domains are required for cooperativety between MEF-2C and myogenic bHLH proteins. Specifically, DNA binding by MEF-2C is not an essential prerequisite for synergism, while the ability to form dimers is a requirement.

Although DNA binding by MEF-2C was not required for synergism between myogenic bHLH factors and MEF-2C, the myogenic bHLH proteins must be bound to DNA via the E-box to active myogenesis in 10T1/2 cells (Molkentin et al., 1995). Myogenin basic region mutants, which lack the ability to bind DNA, failed to activate myogenic conversion of 10T1/2 cell in the absence or presence of wild type MEF-2C.

Early studies demonstrated that two amino acids, alanine114 and threonine115, in the center of the DNA binding domain of myogenic bHLH proteins are required for muscle specific gene activation (Davis, 1992). The placement of asparagines, which are found in corresponding positions of E12, for these alanine and threonine residues resulted in the loss of ability to activate myogenesis while still retaining the ability to bind the E box. Interestingly, if alanine and threonine were introduced into the corresponding positions of the E12 DNA binding domain, E12 gained the ability to active muscle gene transcription (Davis, 1992; Weintraub et al., 1991). Since these myogenic amino acids had no affect on DNA binding, but are essential for myogenesis, it was theorized that these amino acids mediate an interaction with a cofactor which is required for muscle gene activation. Both the mutant MyoD-E12basic, which contains the basic region of E12 in place of the MyoD basic region, and MyoD-E12basic (AT) which has alanine and threonine reintroduced into the E12 basic region were tested for the ability to cooperate with MEF-2C (Molkentin et al., 1995). Only the MyoD-E12basic (AT) had the ability to synergize with MEF-2C. This demonstrated that these specific amino acids in the DNA binding region of MyoD are required for coopererativity with MEF-2C. One possible conclusion from these results is that MEF-2C is the cofactor that is required for the MyoD family to activate muscle gene expression.

MEF2 factors function as homodimers and heterodimers and interact with myogenic bHLH factors to regulate muscle gene expression (Figure Intro-2). In addition to synergizing expression with members of the MyoD family at sites containing E-boxes, wild type MEF-2C and MEF-2A activate transcription through the MEF2 site (Amacher et al., 1993; Black et al., 1995; Cheng et al., 1993; Li and Paulin, 1993; Martin et al.,

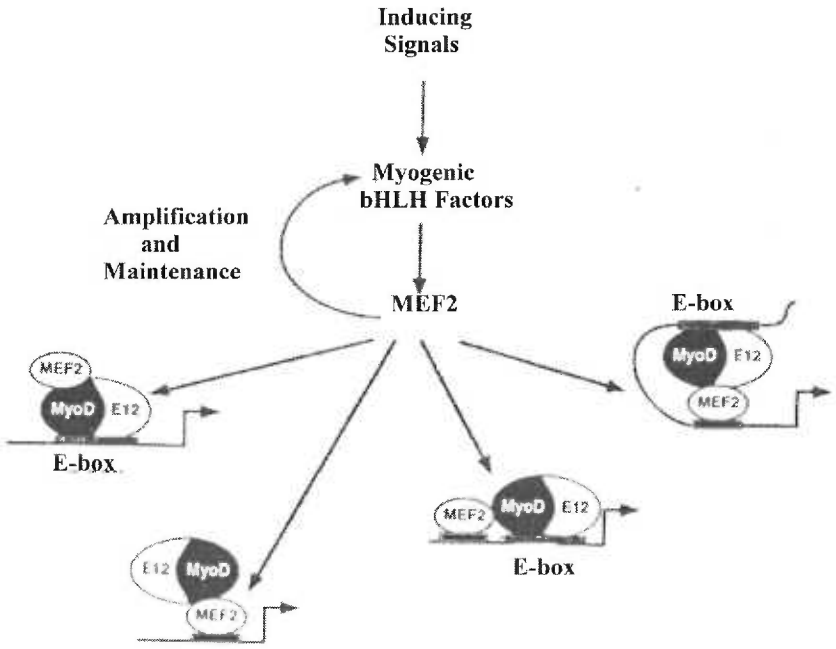

Figure Intro-2. Possible Mechanisms for Muscle Gene Activation
Myogenic bHLH factors are expressed in myoblasts in response to an inducing signal. Myogenic factors can induce MEF2 expression. MEF2 binds the control region of several myogenic bHLH genes, amplifing and maintaining their expression. The Myogenic bHLH proteins activate E-box-dependent genes that also contain MEF2 sites. MEF2 can also act indirectly to activate E-box-dependent genes by interaction with myogenic bHLH proteins. MEF2 can also regulate expression of genes which lack E-boxes in their control regions. Taken in part from Cell, Vol.83, 1125-1136, December 29, 1995.

1993). Dominant negative mutants defective in DNA binding interfere with the ability of wild type MEF-2C to activate transcription at the MEF2 site by sequestering MEF-2C from DNA binding (Molkentin et al., 1995). Alone the MEF2 DNA binding mutants fail to activate MEF2 site transcription but have the ability to activate transcription at the MEF2 independent site by wild type MEF-2C. These mutants therefore retain the ability to synergize with the bHLH region of MyoD and myogenin to activate E-box dependent transcription. This suggests that MEF-2C has the ability to activate two classes of genes. MEF2 proteins can activate transcription in control regions that lack the E-box through direct binding to the MEF2 site. Conversely, MEF2 proteins have the ability to activate

transcription at sites, which lack the MEF2 site, but possess E-boxes. Activation of E-box dependent transcription requires a direct protein-protein interaction with DNA bound myogenic bHLH proteins.

Furthermore, it appears that only the N-terminal 117 amino acids from MEF-2C, which lacks the ability to activate MEF2 site transcription, can effectively activate MEF2 dependent transcription in the presence of myogenic bHLH proteins (Molkentin et al., 1995). In this context, myogenic bHLH factors have the ability to function as transcriptional enhancers for expression at MEF2 sites. This would essentially allow for a greater degree of control by myogenic bHLH proteins in coordinating the establishment of the muscle phenotype in a developing embryo.

Muscle Differentiation and the Cell Cycle

The process of muscle differentiation involves the induction of muscle-specific gene expression which is coupled with withdrawal from the cell cycle and fusion of the myocytes into multinucleated myotubes (Nadal-Ginard, 1978). Given that differentiated myotubes permanently withdraw from the cell cycle, one might theorize that some integration between the function of the myogenic bHLH proteins and a blockade of the cell cycle exists. Since both MyoD and myf-5 are expressed in proliferating myoblasts before terminal differentiation has occurred (Braun et al., 1989; Braun et al., 1989; Davis et al., 1987), there must be some mechanism that modulates the activity and function of these factors to permit cellular proliferation. Differentiation of skeletal myoblasts is dependent on withdrawal from the cell cycle and entry into the G1/G0 phase. In tissue

culture, differentiation is generally initiated by depletion of serum, while the exact mechanism that facilitates myotube formation in vivo remain unknown. Transforming growth factor β(TGF-β) and fibroblast growth factor (FGF) can arrest myoblast differentiation while failing to activate a proliferative response (Hardy et al., 1993). This suggests that the G1/G0 phase of the cell cycle may be composed of multiple sub-compartments, some of which are refractory to the muscle differentiation pathway. In addition, inhibition of myogenesis by activated N-ras and H-ras also lacks continued cell proliferation (Payne et al., 1987).

Growth Factors Inhibit Muscle Development

Growth factors appear to act at multiple levels to inhibit myogenesis. The transcription of genes coding for myogenic determination factors and MyoD is effectively inhibited by growth factors (Brennan et al., 1991; Menko and Boettinger D., 1987). In addition to inhibition of transcription of these genes, growth factors have been shown to inhibit the activity of the myogenin protein (Brennan et al., 1991). High concentrations of mitogens or activated oncogenes such as ras and fos can extinguish the action of muscle-specific bHLH proteins and prevent myogenesis (Lassar et al., 1989; Li Li, 1992). It appears that the constitutive expression of MyoD in proliferating myoblasts prior to differentiation must become activated in some manner upon withdrawal of exogenous growth factors.

The exact mechanism through which growth factors suppress the activity remains unclear. One possibility is that the negative effects of growth factors on myogenesis may

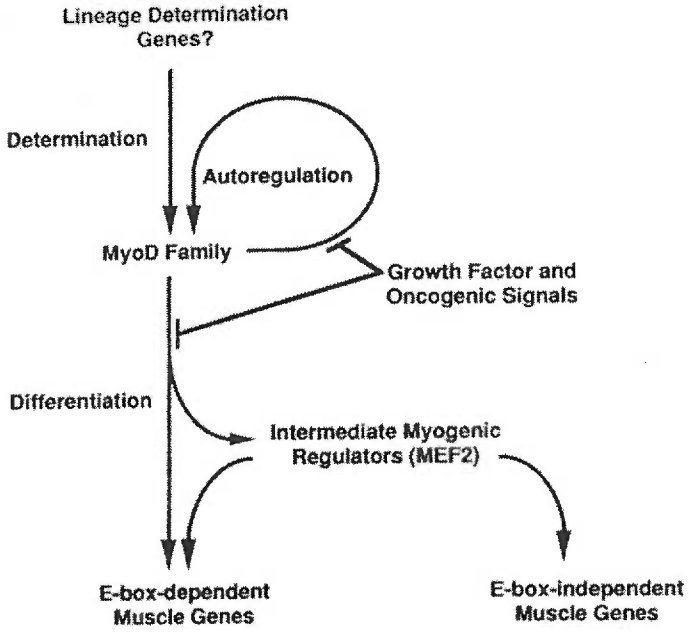

Figure Intro-3 Hypothetical Hierarchy of Regulatory Genes in Myogenic Differentiation
One or more lineage determining genes may be activated in mesodermal progenitor cells, which leads to the formation of myoblasts. Members of the MyoD family are expressed in myoblasts and are able to positively regulate their own expression. Growth factors and oncogene products prevent the activation of genes associated with terminal differentiation and inhibit the activity of the MyoD family. Upon release from growth factor repression, members of the MyoD family activate expression of intermediate myogenic regulators, such as MEF2. Taken in part from Developmental Biology 154, 261-272 (1992).

be mediated through growth factor mediated induction of immediate early gene products. A variety of growth related signaling pathways lead to the induction of c-fos, junB, junC, and c-myc (Lewin, 1991). Repression by Fos and Jun is targeted at the E-box within the MCK enhancer, but does not require direct DNA binding of Fos or Jun to the site. Specifically, the Fos and Jun inhibition of myogenin and MyoD targets the bHLH region (Lassar et al., 1989). It has not yet been determined whether DNA binding by the myogenic bHLH domain was inhibited or if competition for interaction with a factor

which normally interacts with the bHLH regions was being modulated by Fos and Jun expression.

The gene c-myc has also been implicated in the repression of myogenesis by growth factors (Dmitrovsky et al., 1986; Schneider et al., 1987). During myogenesis, c-myc down-regulation occurs prior to the induction of muscle-specific genes. Transfection of established muscle cell lines with c-myc could abolish muscle specific gene expression. Because the Myc protein does not appear to interact with members of the MyoD family or with E2A gene products, it seems its effects are indirect in nature. The HLH proteins Max and Myn were demonstrated to form heterodimers with Myc that effectively bind the CANNTG consensus indicating that Myc may directly induce or repress expression of specific genes (Blackwell, 1990). It appears that the ability of a myoblast to differentiate may be determined by a balance between growth factor generated signals and the differentiation modulating signals mediated by the MyoD family.

Although many growth factors negatively affect muscle differentiation in vitro, several studies suggest that muscle differentiation may be subject to positive regulation by environmental cues. It has been suspected for some time that interaction of integrins with the extracellular matrix is necessary to promote muscle differentiation in culture (Menko and Boettinger D., 1987). In addition, the levels of endogenous insulin-like growth factor (IGF) have been positively correlated with spontaneous muscle differentiation (Montarras D, 1993). Likewise, work done with inducible anti-sense IGF constructs suggest that IGF-1 is required for muscle differentiation in vitro (Powell-Braxton et al., 1993). Also, gene disruption experiments with IGF-1 and the IGF receptor

resulted in mice with hypoplastic muscle, suggesting that IGF may play a role in governing the proliferative action of skeletal muscle stem cells (Liu et al., 1993).

The HLH protein Id Inhibits Muscle Differentiation

During the development of the peripheral nervous system in Drosphila, bHLH genes daughterless and achaete/scute are required for neurogenesis (Jan, 1993). The activity of these genes is opposed by another HLH gene, extramacrochaetae that functions by forming dimers with the achaete and scute genes. The biochemical/structural equivalent of extramacrochaetae was found to exist in the mammalian muscle system, and was termed Id for inhibition of differentiation (Benezra et al., 1990). Members of the Id family contain the HLH motif but lack the basic region and therefore are incapable of binding DNA sequences. Recently it was demonstrated that Id could form heterodimer complexes with MyoD, which lack the ability to bind DNA (Fairman et al., 1993). Likewise, the Id protein possesses the ability to bind E proteins and thereby sequesters the obligate myogenic bHLH dimerization partners. Proliferating myoblasts contain high levels of Id that decrease upon differentiation (Jen Y, 1992). It has been suggested that high levels of Id may prevent MyoD function in proliferating cells. Consistent with this hypothesis, forced expression of Id in myoblasts delayed their differentiation (Jen Y, 1992). It appeared that the Id protein becomes labile under differentiation conditions, and allows for differentiation to occur, but at a markedly slower rate.

Additional evidence that Id plays a physiological role in controlling muscle differentiation came from experiments using chimeric proteins containing both MyoD

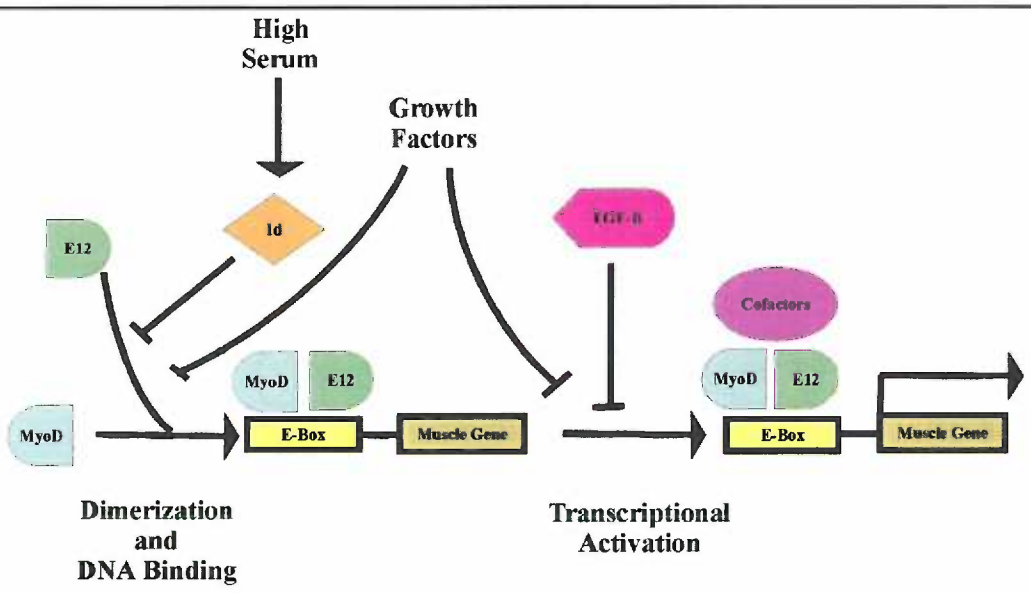

Figure Intro-4 Points of Negative Control of Myogenic bHLH Proteins
Myogenic bHLH proteins (MyoD) dimerize with E12. Id which is induced by serum, also dimerizes with E12 and to a lesser degree MyoD, resulting in inhibition of muscle gene expression. Serum may also inhibit DNA binding by myogenic bHLH factors and E12 proteins, possibly through inducing phosphorylation of the dimerizes proteins. Activation of muscle-specific transcription by myogenic bHLH proteins requires collaboration with other transcription factors and cofactors, such as p300 and MEF2. These cofactors could potentially be repressed by growth factors resulting in failure of the MyoD-E12 complex to bind DNA.

and E proteins connected by a flexible linker (Neuhold and Wold, 1993). This work revealed that the intramolecular HLH interactions were favored over the intermolecular HLH interactions. The chimeric MyoD-E protein bound DNA more avidly and displayed a greater resistance to inhibition by Id protein than did wild type MyoD in electrophoretic mobility shift assays. Although the tethered MyoD-E proteins displayed an increase in functional activity in proliferating cells, the activity of these constructs in the proliferative cells was still diminished relative to their activity in growth arrested cells. These findings support the notion that Id inhibits MyoD function during proliferation and suggest that additional Id independent mechanisms of inhibiting MyoD must exist (Figure Intro-4). Consistent with this prediction, a recent report demonstrated the MyoD-E protein-E-box complexes were not observed in nuclear extracts from proliferating myoblasts (Jen Y,

1992). In other studies, MyoD –E protein complexes were capable of binding to an E-box oligomer in nuclear extracts from both proliferating myoblasts and differentiated myotubes (Simon and Burden, 1993). This raises the possibility that other factors or post-translational modifications that are independent of DNA interactions act as regulators of myogenic bHLH function.

Cyclins Inhibit MyoD Transcriptional Activity

Negative regulation of MyoD function was recently demonstrated by ectopic expression of cyclins D1 and D2 (Skapek et al., 1995). 10T1/2 fibroblasts were co-transfected with plasmids encoding MyoD, cyclin D1, and a reporter MCK-CAT. Expression of cyclin D1 inhibited transactivation of MCK-CAT whereas expression of either cyclins A end E only had mild inhibitory effects. In addition, the retinoblastoma gene product (Rb) was shown to be required for terminal cell cycle withdrawal in SAOS2 osteosarcoma cells (Gu et al., 1993). Given that the ability of Rb to control cell cycle arrest is governed in a negative fashion by G1-cyclin dependent phosphorylation (Chen et al., 1989; Furukawa et al., 1990; Stein et al., 1990), the inhibitor effects of cyclin D1 expression might be mediated through phosphorylation of the Rb protein. Because cyclins A and E, both of which can phosphorylate and inactivate Rb, failed to inhibit MyoD transactivation of MCK-CAT, Rb inactivation may not be sufficient to inhibit MyoD function (Skapek et al., 1995). Furthermore, transcriptional activation of the MyoD right binding site aligned as four tandem repeats upstream of the thymidine kinase

(tk) promoter (4Rtk-CAT) was similarly inhibited by expression of cyclin D1. This suggested that the target of cyclin D1 is the MyoD- E protein heterodimer.

Although cyclin D1 expression did not alter the amount of MyoD protein present in the transfected cells, the electrophoretic mobility of MyoD was affected (Skapek et al., 1995). The change in MyoD mobility in SDS-polyacrylamide electrophoresis was consistent with hyperphosphorylation as seen in MyoD extracted from proliferating cells. In proliferating C2C12 myoblasts, cyclin D1 levels are high and decrease upon differentiation. The catalytic partner for cyclin D1, CDK4 is expressed in both myoblasts and myotubes suggesting that activation of CDK4 may lead to repression of MyoD function (Fiddler, unpublished ; Lees, 1995). Furthermore, data from co-transfection experiments in 10T1/2 suggest that p21 is a potent inhibitor of several cyclin dependent kinases including CDK4 (Halevy et al., 1995; Parker et al., 1995). Ectopic expression of p21 reversed the D1 inhibition of MyoD dependent transcription of the MCK-CAT reporter without altering cyclin D1 protein levels in 10T1/2 cells. In addition, co-expression of both p21 and p16 in C2C12 myoblasts achieved expression from the MCK-CAT reporter equal to levels achieved by culture differentiation conditions of serum starvation (Skapek et al., 1995). Expression of the endogenous muscle gene, myosin heavy chain (MHC) was observed to be 2-3 fold higher in C2C12 cells transfected with both p21 and p16 constructs. These findings indicate that coordinate cell cycle withdrawal and muscle differentiation can be down-regulated through cyclin D1 associated CDK activity.

Oncogenes Inhibit Muscle Differentiation

Myogenic differentiation can be blocked and cell cycle progression promoted by the expression of viral oncogenes (Coppola and Cole, 1986; Dmitrovsky et al., 1986; Falcone et al., 1985; Fiddler et al., 1996; Freytag, 1988; Lassar et al., 1989; Li et al., 1992; Li Li, 1992; Webster et al., 1988). Specific viral oncogenes have the ability to inactivate the retinoblastoma family of tumor suppressor proteins (Sandmoller et al., 1996; Webster et al., 1988; Xiao et al., 1995). High levels of nuclear, hypophosphorylated Rb was observed upon muscle differentiation (Endo and Goto, 1992), implying that Rb may play a role in skeletal muscle differentiation. Moreover, expression of the SV40 large T antigen, which inactivates Rb, drives new rounds of DNA synthesis in otherwise terminally differentiated myocytes (Gu et al., 1993). Contrary to the hypothesis that ectopic expression of MyoD in cells that lack the Rb gene effectively undergo muscle differentiation (Novitch et al., 1996). In contrast to wild type differentiated myotubes which withdrawal from the cell cycle, differentiated myotubes lacking Rb can initiate DNA synthesis and over-expression of the Rb related gene, p107, rescues this defect (Novitch et al., 1996; Schneider et al., 1994). Thus it appears that Rb plays a role in maintaining differentiation through inhibition of DNA synthesis. The fact that Rb deficient myotubes can differentiate indicates that Rb function is not required to support skeletal muscle gene expression. However it is a possibility that functional redundancy within the Rb family may resuscitate the functions governed by Rb during muscle differentiation.

The ubiquitous expression of Rb in normal cells and tissue types suggests that Rb plays essential roles in cell cycle regulation (Coppola et al., 1990; Furukawa et al., 1990). In the G0/G1 phase of the cell cycle, pRb is present in an unphosphorylated form, whereas during the S and M phases it is detected as multiple phosphorylated species (Chen et al., 1989). The unphosphorylated Rb protein associates with MyoD and is essential for MyoD suppression of proliferation and myogenic activation (Gu et al., 1993). The pRb protein also forms complexes with the cellular transcription factor E2F (Helin et al., 1992; Hiebert et al., 1992), c-Myc, and N-Myc (Picksley and Lane, 1994), which are involved in cell proliferation. These finding suggest that unphosphorylated pRb exerts its growth-suppressing effect by two distinct mechanisms: 1) by sequestering the growth promoting transcription factors 2) binding and activating differentiation promoting bHLH factors.

Transcriptional activation of muscle-specific genes by MyoD can be inhibited by expression of the adenovirus E1A gene (Webster et al., 1988). The E1A gene product was recently shown to interact with the p300 transcriptional adapter protein and inhibits its ability to enhance MyoD dependent transcription (Puri Pieri, 1997; Sandmoller et al., 1996). Two domains of p300, at its amino and carboxyl terminals independently function to mediate both co-activation and physical interaction with MyoD (Eckner et al., 1996; Yuan, 1996). A truncated segment of p300, unable to bind MyoD, acts as a dominant negative protein and abrogates both myogenic conversion and transactivation by MyoD, suggesting that endogenous p300 is a required coactivator for MyoD function. Direct protein- protein interaction between p300 and both MyoD and MEF-2C was recently demonstrated suggesting that p300 may act as a linker between the bHLH myogenic

factor and the MEF2 family in coordinated muscle differentiation (Sartorelli, 1997). Thus, inhibition of skeletal myogenesis by oncoproteins may rely upon inactivation of a number of cellular proteins.

Progression through the cell cycle is tightly regulated by a series of positive and negative signals. Disturbances in these signaling pathways may be a significant contributor to the dysregulation of cell growth that is characteristic of malignant cells (Hollstein et al., 1991). The p53 tumor suppressor protein is a sequence specific DNA binding protein and transcription factor and plays a critical role in prevention of malignancies in both men and mouse (Ko and Prives, 1996; Picksley and Lane, 1994; Zambetti et al., 1992). The ability of p53 to prevent malignancies is likely due to its ability to regulate the cell cycle and the apoptotic pathway.

MDM2 Inactivates Tumor Suppressor Products Rb and p53

In animals and cell culture, DNA damage or events that result in abnormal proliferation cause an increase in p53 mRNA and protein levels and p53 dependent transcription (Di-Leonardo et al., 1994; Zambetti et al., 1992). The increased level of p53 protein results in either cell cycle arrest or apoptosis. The biological activity of p53 pertaining to cell cycle arrest stems from p53's ability to activate transcription of the cyclin-dependent- kinase (CDK) inhibitor, p21 (Macleod et al., 1995; Michieli et al., 1994). The p21 protein functions to inactivate growth promoting kinases such as, CDK4

(Halevy et al., 1995; Parker et al., 1995). Other than inducing BAX expression, is still unclear as to how p53 regulates the apoptosis pathway.

Recently, an endogenous gene product was identified that can bind to the p53 protein and affect its function (Oliner et al., 1992). The MDM2 protein, initially identified as an oncogene involved in murine transformation (Fakharzadeh et al., 1991), was demonstrated to bind to the p53 protein and inhibit p53 mediated transcription without effecting p53 protein levels (Chen et al., 1993; Momand et al., 1992; Oliner et al., 1992; Oliner et al., 1993). Inhibition of p53 function is thought to be due to MDM2 binding to the region of p53, which acts as a transcriptional activator. In cells lines that are capable of undergoing p53 mediated apoptosis, overexpression of MDM2 efficiently inhibited apoptosis (Chen et al., 1996). Initially, it was shown that MDM2 must be able to bind to the p53 protein in order to abrogate p53 mediated cell cycle arrest (Chen et al., 1996; Haines et al., 1994; Sisalas et al., 1996). Additional mutational analyzes revealed that MDM2 mutants which lacked the ability to bind the p53 protein still retained the ability to inhibit p53 dependent cell cycle withdrawal (Sisalas et al., 1996). Interestingly, the MDM2 proteins have been found at high levels in murine and human tumor samples (Forus et al., 1993; Khatib et al., 1993; Kondo S, 1996; Nilbert et al., 1994; Reifenberger et al., 1994). It appears that the multiple forms of MDM2 are the result of alternative splicing of the MDM2 transcript. Transfection experiments using these natural MDM2 variants identified alternatively spliced MDM2 products that failed to bind p53, but still retained the ability to transform 3T3 cells (Sisalas et al., 1996). This suggests that multiple mechanisms must exist, p53 dependent and p53 independent, by which MDM2 can inhibit cell cycle arrest.

Analysis of the structure of the mdm2 gene revealed the presence of p53 binding sites within the first intron of the mdm2 gene (Barak et al., 1993; de-Oca-Luna et al., 1996). Although the p53 sites are imperfect consensus sites, expression of p53 results in an increase in both MDM2 transcripts and protein levels. Because MDM2 protein can combine with p53 and negatively modulate its activity as a transcription factor, the regulation of the mdm-2 gene by p53 protein has interesting consequences. When MDM2 is expressed in a cell where p53 is active, it blocks further p53 function leading to a reduction in MDM2 levels. Thus the activity of p53 and the levels of MDM2 in a cell are kept in balance by an auto-regulatory loop. Factors that disturb this loop, such as MDM2 gene amplification, or increase MDM2 activity will result in cellular proliferation (Fiddler et al., 1996; Wu et al., 1993). Alternatively, situations that alter the ability of p53 protein to stimulate MDM2 or inactivate MDM2 should lead to growth arrest. In fact, mdm2 null mice are not viable and die during gestation, while the double p53/mdm2 knockout survives to adulthood (Jones et al., 1995; Montes-de-Oca-Luna et al., 1995). This suggests an essential role of MDM2 in control of the growth inhibitory effects of p53.

Likewise, the p53-binding domain of MDM2 has been shown to inhibit the G1 blocking effects of p107 and pRb (Lundgren K et al., 1997; Xiao et al., 1995), and to interact with the S-phase promoting E2F/DP1 complex (Martin et al., 1995). Expression of MDM2 in U2OS cells resulted in a stimulation of E2F/DP1 dependent transcription from CAT constructs driven by E2F/DP1 binding sites (Martin et al., 1995). Since E2F-1 responsive genes encode products that are important for induction of S phase (DeGregori et al., 1995; Lam and La-Thangue, 1994), stimulation of E2F/DP1 transcription factor

complex activity by the binding of MDM2 to either E2F and DP1 subunits or Rb suggests a p53 independent role for MDM2 in promoting cell growth.

Rhabdomyosarcomas are one of the most common solid tumors of childhood. Sarcomas have traditionally been classified as rhabdomyosarcomas based on morphology and expression of muscle structural genes, such as myosin heavy chain or desmin. Recently, expression of MyoD has been shown to be the most sensitive marker for classifying sarcomas as rhabdomyosarcomas (Mitchell et al., 1991; Pereira-Smith and Smith, 1988; Shimizu et al., 1990). This is paradoxical because MyoD has been shown to induce muscle differentiation in a wide variety of primary and transformed cell lines (Weintraub et al., 1989), yet rhabdomyosarcomas have a low propensity to differentiate into myotubes. Thus, even though MyoD is expressed in rhabdomyosarcoma cells, it would appear to be non-functional in inducing differentiation of these cells. The purpose of my graduate research, compiled in this dissertation, was to gain a more in-depth understanding as to the mechanism governing the failure to differentiate into mature myotubes seen in rhabdomyosarcoma tumors. Briefly, amplification and over-expression of MDM2 results in results in the failure to activate muscle-specific gene transcription. Furthermore, repression of gene transcription appears to be DNA site specific and mediated through two separate pathways: 1) Rb and 2) MEF-2C.

Materials and Methods

Cells

C2C12 cells and 10T1/2 cells were obtained from the American Type Culture Collection. Rh18 and Rh30 cells were provided by P. Houghton. All cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% calf serum (Hyclone Laboratories). Rh18 cells were stably transfected with pRSVNEO by electroporation (300 volts, 960 μFD in PBS) (BioRad), and approximately 2,000 clones were pooled and expanded for use as donors in microcell fusions. Myogenic differentiation was induced by growing cells to confluence followed by incubation in DMEM with 2% horse serum (differentiation medium).

Microcell Mediated Chromosome Transfer

Rh18 cells, or the C2 (Rh18) primary microcell hybrids, were micronucleated by adding 10.0 μg or 0.06 μg, respectively, of colcemid per ml in DMEM plus 15% calf serum for 48 hr. The micronucleate cell populations were enucleated by centrifugation in the presence of 5μg of cytochalasin B (Sigma) per ml, and the isolated microcells were fused to C2C12 recipients as described (Fournier, 1981; Lugo et al., 1987) . Microcell hybrid clones were isolated using cloning cylinders after 3 to 4 weeks of selection in medium containing 500 μg of Geneticin (Gibco) per ml.

Cell Transfections

Cells were transiently transfected by the LipofectAMINE (GibcoBRL) method. Approximately 3 X 10^5 cells were plated 1 day prior to transfection into 60mm tissue culture plates. The day of transfection cells were washed twice with serum free DMEM. Transfection mixtures were comprised of a total DNA content of 6 μg with 0.5 - 1.0 μg being represented by the reporter construct. The remaining amount of DNA was comprised of other constructs mentioned in the text. The lipid-DNA mixtures were added to the washed cells and brought to a final volume of 2 milliliters with serum free DMEM. The transfection mixture was allowed to sit on the cells for 6 hours after which the transfection solution was removed by aspiration. DMEM containing 15% calf serum was added to the cells, and the cells harvested after approximately 48 hrs.

CAT Assays

CAT activity was measured using a phase extraction procedure (Seed and Sheen, 1988) . In brief, 48 hours after transfection, cell extracts were generated by freeze-thawing cell pellets in 100 μl 0.25 M Tris HCl (pH 7.5). Following treatment at 65°C for 15 min. to inactivate endogenous acetylases, 30 μl of extract was assayed with 0.2 mCi of (^{3}H) chloramphenicol (Dupont-New England Nuclear) and 250 nM butyryl-CoA (Sigma), in a total volume of 100 μl. The reaction was allowed to proceed for 2-12 hours at 37°C and stopped by mixing with 200 μl TMPD-Xylene (2:1) (Sigma), and then 130 μl of the upper phase was added to scintillation cocktail and counted. Results are presented in percentage activity or fold activation with error bars indicating standard deviation.

Northern Analysis

Total cytoplasmic RNA was prepared as described (Favaloro et al., 1980) . Five micrograms total cytoplasmic RNA was used for Northern analysis on 1.5% agarose gels containing 6.7% formaldehyde. RNA was transferred to GeneScreen (Dupont) by capillary transfer in 10x SSC (1x SSC is 150 mM NaCl, 15 mM Na-citrate). RNA was cross-linked to the membrane by exposure to UV, followed by baking at 80^{o}C for 2-4hr. Blots were prehybridized for several hours at 42^{o}C in hybridization buffer (50% formamide, 1% bovine serum albumin {fraction V}, 1mM EDTA, 0.5M sodium phosphate {pH 7.2}, 5% sodium dodecyl sulfate {SDS}). Hybridizations were for 24hr at 42^{o}C in fresh hybridization buffer containing 1 x 10^{8}cpm of randomly primed {^{32}P}-labeled DNA probe. The filters were washed in 2X SSC-0.1% SDS for 15min at room temperature, in 0.1X SSC-0.1% SDS for 15min at room temperature, and in two changes of 0.1X SSC-0.1% SDS at 55^{o}C for 15min each. The blots were stripped for reuse by boiling for 2 min. in double-distilled water.

Southern Analysis

High molecular weight DNA (10 μg) was digested to completion with Hind III (New England Biolabs) and separated on 0.8% agarose gels in 0.04 M Tris acetate-2 mM EDTA. The DNA was capillary transferred in 10x SSC (1x SSC is 150 mM NaCl, 15 mM Na-citrate) to Gene Screen (Dupont) membranes, and UV crosslinked as described (Church and Gilbert, 1984) . The blots were prehybridized and hybridized as for

Northern blots. Probes containing Alu sequences were processed and hybridized as described by (Budowle and Baechtel, 1990) to minimize background from repetitive sequences. The blots were stripped for reuse in 0.2N NaOH for 30 min at 65 ^{o}C.

Inter Alu PCR

The PCR was carried out in a total volume of 50μl with 1μg of DNA, primer at 1μM in 50mM KCl, 10mM Tris HCl, pH8.0, 1.5mMMgCl_2, 0.01% gelatin, 200 μM dCTP, dATP, dGTP, dTTP (Pharmacia), and 1 unit Taq polymerase (Cetus) for 30 cycles of 94^{o}C denaturation (1min), 60^{o}C annealing (30sec), and 72^{o}C extension (30sec) in an automated thermal cycler (Cetus). The reaction was carried out in the presence of a single Alu primer, 517 (Nelson et al., 1989) .

Fluorescent In Situ Hybridization

DNA probes were nick-translated using standard protocols to incorporate biotin-11-dUTP or digoxigenin-dUTP. Slides of chromosomally normal male metaphase spreads were obtained from peripheral blood (Yunis and Chandler, 1978) . Hybridizations were carried out on slides at 37^{o}C for 16 hrs. Final probe concentrations varied from 40-60ng/μl. Signal detection was carried out according to Trask and Pinkel (Trask and Pinkel, 1990) . Amplification of the biotinylated probe signal utilized alternating incubations of slides with anti-avidin (Vector) and FITC-Extravidin (Sigma). Amplification of digioxygenated probes utilized alternating incubations of slides with FITC-tagged sheep antibodies made in rabbit and FITC-tagged rabbit antibodies made in sheep (Boehringer Mannheim).

Slides were counterstained with propidium iodide (0.3μg/ml), coverslipped, and viewed under UV fluorescence with FITC filters (Zeiss). Metaphase spreads showing probe signal were photographed with Fuji color film (ASA 100) at ASA 400. Identification of chromosomal loci were obtained by sequentially staining the same metaphase spreads with chromomycin A3/distamycin to produce fluorescent R-bands. R-banded metaphase spreads were then photographed with technical pan 2415 film (Kodak) at ASA 400.

Cell extracts

Cultured cells were grown in DMEM plus 15% calf serum (Hyclone) and were harvested at about 90% confluence. Plates were washed three times with Tris-buffered saline solution, dislodged by scraping and pelleted by centrifugation for 15 seconds at 14K rpm. Cells were resuspended 0.5 ml/dish of lysis buffer (20mM HEPES {pH 7.6}, 20% glycerol, 0.5 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5% NP40, 1 mM DTT, 1 mM PMSF, 10 μg /ml leupeptin, 10μg /ml pepstatin, and 100μg /ml aproteinin). Lysed cells were vortexed occasionally for 15 min. at 4°C and centrifuged at 14K rpm for 15 min., and supernatant was dialyzed extensively against lysis buffer containing only 10mM NaCl instead of 0.5 M NaCl.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assays were performed essentially as described (12). Purified proteins were incubated with or without 1-10μg cellular extract in a total volume of 10μl. DNA-binding reactions were for 5 min. at room temperature in 20mM HEPES

(pH 7.6, 50mM KCl, 3mM $MgCl_2$, 1 mM EDTA, 0.5% NP-40 and 500 ng poly(dI-dC) in a final volume of 20 µl. The mixtures were loaded onto a 2% agarose gel (Seaplaque low-melting; FMC) containing 50mM Tris base, 50mM boric acid, and 1mM EDTA (same solution in running buffer) and run at room temperature or 4°C as indicated. Gels were visualized by exposure to Kodak X-Ray film.

In vitro Translation and Electrophoretic Mobility Shift Assay

In vitro translations were performed by using rabbit reticulocyte lysate system (Promega TNT) in a 50 µl mixture containing 0.5 - 1.0µg of circularized vector, 25µl of reticulocyte lysate, supplement amino acids (1mM each), 70mM KCl, 10µCi ^{35}S-methionine (New England Nuclear,), 2.4mM $MgCl_2$, and 0.5U/µl RNasin (Promega) at 30°C for 60min. The ^{35}S labeled proteins were resolved on a 5 – 10% SDS-polyacrylamide gel. Prestained molecular weight markers were used to determine the size of labeled proteins.

In vitro Binding Assay, Immunoprecipitation, and coimmunoprecipitation.

Labeled proteins prepared from the reticulocyte lysate system and incubated at the desired temperature (4°C to 37°C) for 5 to 30 minutes. Incubation with antisera against MDM2 (SMP14 Santa Cruz), MDM2 (N-20 Santa Cruz), MyoD (NP-40), HA was performed. The complex proteins were then washed three times in RIPA buffer (50mM Tris-HCl {pH 7.7}, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate,).1% SDS) containing 1mM phenylmethylsulfonyl fluoride and 10µg each of aprotinin and leupeptin

per ml. Samples were placed in 10μl loading buffer (1M Tris{pH 6.8}, 20% glycerol, 0.02% bromophenol blue, 5% SDS, 10% 2-mercaptoethanol) and boiled for 5 minutes. Immunoprecipitaed were resolved on a 5% to 10% denaturing polyacrylamide gel and dried for radiography.

Western Blotting

Cell pellets were lysed in RIPA containing protease inhibitors (as described above). Approximately 100 μg of extract from either asynchronously growing or serum starved cells were resolved in a 8% SDS polyacrylamide gel, and transferred to Immobilon P (Millipore) according to standard procedures. The following antibodies were used for Western blot analysis: MDM2 (SMP14 Santa Cruz), MDM2 (N-20 Santa Cruz), MyoD (NP-40), and anti- HA.

PCR Analysis

High molecular weight DNA (100 ng) was subjected to PCR in the presence of 67mM Tris (pH 8.8), 16mM $(NH_4)_2S0_2$, 10mM 2-mercaptoethanol, 6.7μM EDTA, 2.0mM $MgCl_2$, 10% glycerol, 0.2mM dNTPs, 0.2pM primers, 1.3U Taq polymerase (Perkin-Elmer). Samples were amplified 30 cycles: 95°C 30 sec, 60°C 30 sec, 72°C 30 sec. Samples were electrophoresed on 1.0 % agarose gels and stained with ethidium bromide. Primer sets obtained from Research Genetics were subject to a different protocol, see appendix I for a list of Research Genetics primer sets. Genomic DNA (20 ng) was subjected to PCR in the presence of 67mM Tris (pH 8.8), 16mM $(NH_4)_2S0_2$, 10mM 2-

mercaptoethanol, 6.7μM EDTA, 1.5 $MgCl_2$, 10% glycerol, 0.2mM dNTPs, 0.2pM primers, 0.02U Taq polymerase (Perkin-Elmer) in a 10μl volume. PCR involved three phases: Phase 1 95°C for 2minutes; Phase 2 30 cycles 94°C 30 seconds, 57°C 45 seconds, 72°C 60 seconds; Phase 3 72° 7 minutes. Samples were electrophoresed on 2.5 % agarose gels and stained with ethidium bromide.

Yeast Two Hybrid

Yeast strain L40 was grown in YPAD overnight, shaking at 30°C. The overnight culture was diluted to an OD600 of 0.2 in 50ml YPAD and grown an additional 4 hours. Cells were pelleted at 2500 rpm, room temperature, and resuspended in 40 mls TE. The suspension was repelleted as above and resuspended in 2 mls 0.1M LiAc and incubated at room temperature for 10 minutes. One μg of each plasmid DNA and 100 μg sheared and denatured salmon sperm DNA were placed into 1.7ml tube. One hundred μl of yeast suspension from above was added to each tube and well mixed. Next 700 μl 100mM LiAc/PEG was added to the tubes. Following mixing the tubes were incubated at 30°C for 30 minutes. DMSO (88 μl) was added and the solution mixed and placed at 42°C for 7 minutes. The tubes were microfuged for 10 seconds and the supernatant was aspirated. The pellets were washed with 1.0 ml TE and repelleted, followed by aspiration of the supernatant. Cells were resuspended in 20 μl TE and plated on selective media. Colonies were picked to a grid in 2-3 days and assayed for β-GAL activity.

Immunohistochemistry

Clones were grown to large, dense colonies and placed under serum starvation for 48 hours. Following culture plates were rinsed twice in PBS at room temperature and fixed for 1 minute in ice cold AFA (68% ethanol, 26% water, 3.2% formaldehyde, and 2.8% glacial acetic acid). The fixed cells were then rinsed four times at room temperature in PBS for 5 minutes. Colonies were blocked for 1 hour at room temperature in PBS, plus 1% horse serum. The solution was changed and primary antibody (MF20 MHC) was diluted 1:20 in PBS, plus 1% horse serum and incubated for 1 hour at room temperature. Following incubation plates were washed four times in PBS, 5 minutes each. Secondary antibody was diluted 1:400 in PBS, plus 1% horse serum and incubated at 4°C for 1 hour. Following incubation plates were washed four times with PBS for 5 minutes each wash. Substrate for conjugated secondary antibody was added and colony detection performed.

Chapter 1

Amplification and Overexpression of MDM2 Inhibits MyoD and myf-5 Mediated Myogenesis

Background

Rhabdomyosarcoma are Skeletal Muscle Tumors

Rhabdomyosarcomas are one of the most common solid tumors of childhood. Based on histological and cytogenetic criteria, rhabdomyosarcomas are grouped as either embryonal or alveolar : a balanced translocation between chromosomes 2 and 13, t (2:13)(q35; q14), (Barr et al., 1993) is associated with alveolar rhabdomyosarcomas and a loss of heterozygosity on the short arm of chromosome 11 encompassing 11p15.5 is associated with embryonal rhabdomyosarcomas (Scrable et al., 1990). A recent report has demonstrated that the PAX3 gene is rearranged in the t (2; 13) translocation (Barr et al., 1993; Shapiro et al., 1993). Loss of heterozygosity at 11p15.5 is associated with a number of solid tumors (Newsham et al., 1991), suggesting the location of a tumor suppressor gene, or genes, for multiple tumor types in this region. While abnormalities in p53 and ras have been reported in rhabdomyosarcomas (Hollstein et al., 1991) the loci involved in the loss of heterozygosity have not been identified, although MyoD has been excluded (Dias et al., 1990; Hazelton et al., 1987; Hiti et al., 1989). Recently, chromosome transfer experiments introducing normal chromosome 11 into rhabdomyosarcoma cells have demonstrated inhibition of cell growth and tumor formation in nude mice (Garvin et al., 1986; Loh et al., 1992), but had no effect on myogenic differentiation.

Skeletal muscle differentiation consists of an ordered withdrawal of committed cells from the cell cycle and their fusion to form multinucleated myotubes (Emerson, 1993; Lassar et al., 1994; Yun and Wold, 1996). Under conditions of low mitogenic

stimulus, in vitro, the differentiation of skeletal muscle myoblasts displays withdrawal from the cell cycle and myotube formation. Upon fusion, there is coordinate expression of numerous unlinked genes that encode muscle-specific proteins and accumulation of gene products, which are required for the proper assembly, and function of the contractile apparatus. Recent evidence suggests that commitment to the myogenic lineage is under the control of a subset of regulator genes. Studies regarding myogenic determination have identified a family of genes MyoD (Davis et al., 1987), myogenin (Edmondson and Olson, 1989; Wright et al., 1989), MYF-5 (Braun et al., 1989), and MRF-4 (Braun et al., 1990; Miner and Wold, 1990; Rhodes and Konieczny, 1989) involved in both determination and differentiation of skeletal muscle. MyoD, myogenin, MYF-5, and MRF-4 are expressed exclusively in skeletal muscle and share extensive sequence homology.

Sarcomas have traditionally been classified as rhabdomyosarcomas based on morphology and expression of muscle structural genes, such as myosin heavy chain or desmin. Recently, expression of MyoD has been shown to be the most sensitive marker for classifying sarcomas as rhabdomyosarcomas (Mitchell et al., 1991; Pereira-Smith and Smith, 1988; Shimizu et al., 1990). This is paradoxical because MyoD has been shown to induce muscle differentiation in a wide variety of primary and transformed cell lines (Weintraub et al., 1989), yet rhabdomyosarcomas have a low propensity to differentiate into myotubes. Thus, even though MyoD is expressed in rhabdomyosarcoma cells, it would appear to be non-functional in inducing differentiation of these cells.

MDM2 is Associated with Neoplasia

Various forms of cancer have been shown to contain abnormalities associated with chromosome 12 (Forus et al., 1993; Suijkerbuijk et al., 1994). Specifically, the region 12p12-15 is often the target of DNA amplification events (Forus et al., 1993; Khatib et al., 1993; Meddeb et al., 1996; Nilbert et al., 1994). Genetic mapping of this region has resulted in the localization of multiple cell cycle, cellular differentiation associated genes, and oncogenes. Chromosomal amplification of genes within this region shows preferential involvement of CDK4, SAS, and MDM2 (Reifenberger et al., 1994; Roberts et al., 1989). The occurrence of amplicons containing one or more of these genes varies depending on what type of tumor is characterized. The molecular analysis of primary gliomas revealed that 15 % of tumor samples contained CDK4 gene amplification (Khatib et al., 1993). Amplification of CDK4 was always associated with an increase in CDK4 gene expression. In addition, some tumors which lacked the CDK4 amplification displayed overexpression of the CDK4 gene (Khatib et al., 1993; Reifenberger et al., 1994). Approximately 10 % of the primary tumors tested revealed a 5-50 fold MDM2 gene amplification (Ried et al., 1994). Similar to CDK4, amplification of MDM2 was always associated with an over abundance of MDM2 RNA (Bueso-Ramos CE and Glassman A, 1996). Analysis of osteosarcomas detected MDM2 gene amplification in 15% of the samples tested (Friend et al., 1986; Tarkkanen et al., 1995). Amplification of MDM2 was significantly higher in metastatic and recurrent tumors than

that of primary osteosarcomas (Ladanyi et al., 1993). Studies involving soft tissue sarcomas demonstrated amplification of MDM2 in 15% to 36% of the samples tested (Kinzler et al., 1987). Sarcomas also show a slight increase in the frequencies of MDM2 amplification in metastatic tumors as compared to primary tumor samples. Recently, we have demonstrated MDM2 amplification in primary rhabdomyosarcoma tumor samples of approximately 45 percent.

MDM2 was originally cloned as an amplified gene in the tumorgenic 3T3DM cell line (Cahilly-Snyder et al., 1987; Fakharzadeh et al., 1991), encoding a protein that inhibited p53 function by masking the transcriptional activation domain of p53 (Momand et al., 1992; Oliner et al., 1993). Cell lines over-expressing MDM2 display an inhibition to p53 mediated cell cycle arrest (Chen et al., 1996; Lundgren K et al., 1997). This arrest of p53 function is due in part to the MDM2 negation of p53 dependent transcription. MDM2 binding to the transcriptional activation domain of p53 has recently been shown to occur through the MDM2 N-terminal region (Chen et al., 1993; Haines et al., 1994; Kussie et al., 1996; Momand et al., 1992; Oliner et al., 1993). In normal cells, MDM2 and p53 form a negative feedback loop that helps limit the growth suppressive activity of p53. In events that lead to an increase in p53 expression, the p53 protein can activate expression of the MDM2 gene (Wu et al., 1993; Zambetti et al., 1992). The MDM2 gene in turn can bind the p53 transcriptional activation domain inhibiting further p53 dependent transcription (Oliner et al., 1993). Deletion of MDM2 is lethal in mice (Jones et al., 1995), while p53 deficient mice mature normally. In contrast to the MDM2 knockout model, mouse embryos lacking both MDM2 and p53 are viable (Jones et al.,

1995; Montes-de-Oca-Luna et al., 1995). This suggests that down regulation of the growth suppressing effects of p53 is a key activity of MDM2.

The research compiled into this dissertation was directed towards elucidating the mechanism by which rhabdomyosarcoma cells fail to differentiate into mature muscle fibers. Rhabdomyosarcoma cell lines were characterized and analyzed at a molecular genetic level. Candidate genes were characterized and evaluated for the ability to reproduce the rhabdomyosarcoma cells inability to differentiate. The MDM2 gene was found to be amplified and overexpressed in rhabdomyosarcoma Rh18 cell line. Likewise, expression of MDM2 in mouse myoblast cells extinguished these cells' ability to form mature myotubes.

Results

Rhabdomyosarcomas fail to activate Muscle-Specific Gene Transcription

Recently, analysis of five rhabdomyosarcoma cell lines (RD, Rh18, Rh28, Rh30, and RhJT) for expression and function of the myogenic regulatory gene family was carried out (Tapscott et al., 1993). Four of the five rhabdomyosarcoma cell lines (RD, Rh28, Rh30, and RhJT) express MyoD and myogenin, while the fifth cell line (Rh18) expresses only Myf-5 and myogenin RNA. MyoD was tested for transcriptional activation in these five cell lines. In contrast to a number of other tumor cell lines, MyoD was not able to activate expression of a muscle specific reporter construct 4Rtk-CAT, nor the more complex MCK-CAT in any of these rhabdomyosarcomas. Likewise,

immunohistochemistry stains on transfected cells demonstrated high amounts of nuclear MyoD, but no increase in expression of MHC. Since the functional MyoD complex is thought to contain both MyoD and an E2A protein (Neuhold and Wold, 1993; Thayer and Weintraub, 1993), it was possible that MyoD did not activate transcription because the E2A protein was limiting in supply. While MyoD and E2A act synergistically in a number of cell lines, in the rhabdomyosarcoma cells E2A failed to rescue the ability of MyoD to activate the muscle-specific reporter (Tapscott et al., 1993). Failure of MyoD to activate expression from these muscle-specific constructs could result from either the inability of MyoD to activate transcription or the failure of MyoD-E12 protein complex to bind target DNA sites. To address these issues chimeric proteins were constructed using wild type MyoD, the VP16 transcriptional activation domain, and Gal4 DNA binding motif. Expression of MyoD-VP16 in the rhabdomyosarcoma cells displayed transcriptional activation of muscle constructs 4Rtk-CAT and MCK-CAT ten times that seen with expression with wild type MyoD indicating that MyoD –E12 complex are capable of interacting with DNA (Tapscott et al., 1993). Additional support came from electrophoretic mobility shift assays using nuclear extracts from rhabdomyosarcoma cells and radiolabeled DNA containing the MyoD consensus binding sequence. All rhabdomyosarcoma nuclear extracts exhibited effective MRF binding site activity (Fiddler, unpublished ; Tapscott et al., 1993). The ability for MyoD to function as a transcriptional activator was addressed by the Gal-MyoD fusion constructs ability to activate transcription from the GM4tk-CAT reporter which contains the CAT gene driven by four MyoD sites that alternated with four Gal4 binding sites. Co-transfection of Gal-MyoD and GM4tk-CAT into RD, Rh30, Rh28, and RhJT resulted in activation of the

GM4tk-CAT construct ten to fifteen times that found in 10T1/2 alone (Tapscott et al., 1993). Therefore, MyoD can act as a transcriptional activator in the context of other factors to establish an active transcriptional complex in rhabdomyosarcoma tumor cells, but is somehow challenged in its traditional setting.

Rhabdomyosarcoma Cells Exist as Two Distinct Phenotypes

The inability of MyoD to activate transcription in these rhabdomyosarcomas could be due to either a factor that actively suppresses MyoD function, or due to the absence of a co-factor that is necessary for MyoD function. These two possibilities make different predictions on the behavior of rhabdomyosarcomas in complementation assays. The presence of an activity capable of suppressing MyoD function would be predicted to behave as a dominant trait, while the absence of a MyoD co-factor would therefore behave as a recessive trait. These possibilities were addressed by the fusion of the rhabdomyosarcomas with 10T1/2 cell, and assayed for the muscle phenotype.

Isolation of the Dominant Inhibitory Loci

Heterokaryon formation between 10T1/2 cells and RD, Rh28, Rh30, and RhJT resulted in the differentiation of the heterokaryon into muscle and restoration of the MyoD transcriptional activity (Tapscott et al., 1993). Since 10T1/2 cells can complement the myogenic phenotype in this set of rhabdomyosarcomas, it appears as though a recessive defect is responsible for the non-muscle phenotype observed in RD, Rh28, Rh30, and RhJT. In contrast, neither differentiation nor restoration of MyoD activity resulted from heterokaryon formation between 10T1/2 and Rh18. Furthermore, hybrid

formation between Rh18 and the myoblast cell line C2C12 results in the inhibition of the muscle phenotype. This observation is consistent with a dominant non-differentiating phenotype expressed in the Rh18 rhabdomyosarcoma cell line. Characterization of Rh18, with regard to defects in myogenic differentiation will lead to a greater understanding of muscle cell development and cancer biology.

To study the genetic basis of the dominant inhibitory phenotype of Rh18 cells, we utilized microcell fusion to transfer chromosomes from Rh18 cells into the mouse myoblast cell line C2C12. We chose C2C12 cells as recipients because non-differentiating variants occur at a low frequency (Peterson et al., 1990). Microcell fusion is a technique that can be used to transfer single, intact chromosomes from one mammalian cell to another. Briefly, microcell fusion involves the induction of donor cells to become micronucleated by prolonged mitotic arrest, partitioning the chromosomes into subnuclear packets (micronuclei). The micronuclei are then physically isolated from the cells by centrifugation in the presence of cytochalasin B. The particles that are produced (microcells) consist of a single micronucleus and a thin layer of cytoplasm surrounded by an intact cytoplasmic membrane. Fusion of isolated microcells with intact recipients, and the utilization of appropriate selective conditions, yields proliferating microcell hybrid clones. Unfortunately, relatively few markers exist which can be selectively fixed in cultured cells. However, the advent of cloned, dominant, selectable markers and the ability to introduce these markers by transfection or recombinant retroviral vector, allows one to selectively tag and transfer previously non-selectable chromosomes from virtually any cell line into another.

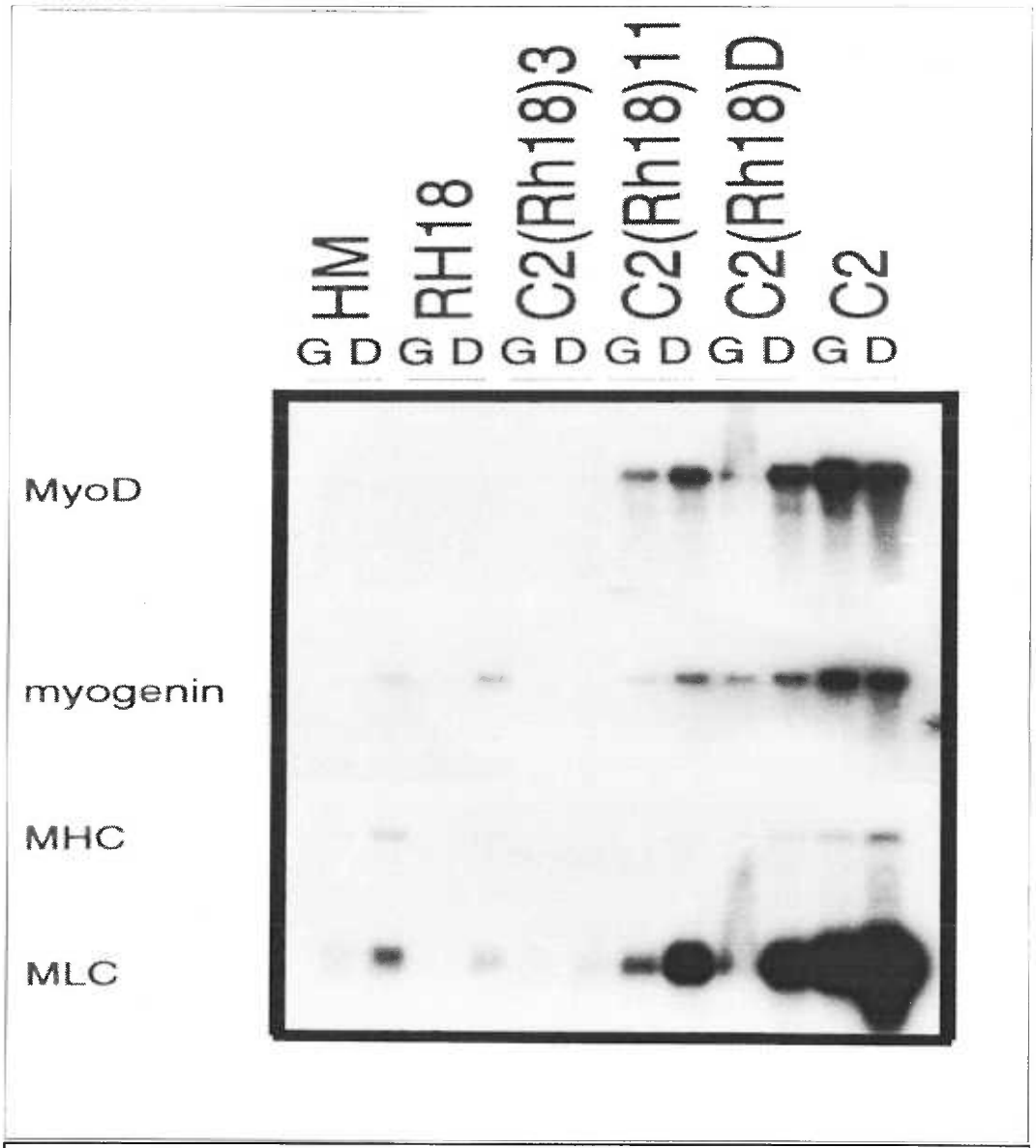

Figure 1-1. Expression of muscle-specific genes in Rh18-C2C12 microcell hybrids. Northern blot analysis of 5$_{\mu}$g of cytoplasmic RNA extracted from cells in growth medium (G) and cells in differentiation medium (D) are shown. Northern blots were probed with cDNAs corresponding to MyoD, myogenin, MHC, and MLC. Ethidium bromide staining of the samples used for the Northern analysis showed equal loading of all samples. HM, human myoblast.

Rh18 chromosomes, randomly tagged by transfection with the plasmid pRSVNEO (neo^r), were transferred into C2C12 cells by microcell fusion. Following

selection in media containing G418, we screened approximately 100 hybrid clones for expression of the muscle phenotype by visual inspection (see Figure 1-2). Ten non-differentiating clones, named the C2 (Rh18) family, were isolated and expanded. All ten clones were assayed for expression of myosin heavy chain (MHC) by indirect immunofluoresence. Three clones, C2 (Rh18)-3, C2 (Rh18)-11, and C2 (Rh18)-D, continued to show greater than 90% reduction in the number of cells expressing MHC when compared to parental C2C12 cells (Thayer, unpublished), and where chosen for further analysis.

Expression of MyoD, myogenin, MHC, and myosin light chain 1/3 (MLC) were assayed by northern blot hybridization. Figure 1-1 shows that clone C2 (Rh18)-3 does not express detectable levels of the myogenic regulatory genes MyoD and myogenin, or the differentiation-specific mRNAs for MHC or myosin light chain 1/3 (MLC). Clones C2 (Rh18)-11 and C2 (Rh18)-D continue to express MyoD and myogenin, albeit at a reduced level, when compared to parental C2C12 cells, and expressed low but detectable levels of MHC and MLC. These results indicate that transfer of Rh18 chromosomes into C2C12 cells can result in at least two different phenotypes, presumably due to retention of different Rh18 chromosomes. One phenotype is characterized by a complete lack of expression of MyoD and myogenin, and a second by continued expression of MyoD and myogenin, both result in greater than 90% reduction in cells expressing muscle differentiation-specific genes.

Since microcell fusion can result in the transfer of more than one chromosome, we next determined the number of human chromosomes retained in the C2 (Rh18) hybrids. We used total human DNA to specifically "paint" human chromosomes in metaphase

spreads from each of the three primary microcell hybrid clones. All three hybrids retain two to three human chromosomes (Thayer,unpublished). Therefore, in order to determine whether the neo^r insertions are located in the chromosomes that contain the dominant inhibitory loci, and to determine the chromosomal map positions of these loci, we conducted a second round of microcell fusions. Each primary microcell hybrid, C2 (Rh18)-3, C2 (Rh18)-11, and C2 (Rh18)-D, was utilized as a donor in microcell fusions with C2C12 cells. Secondary microcell hybrids were generated by selecting for transfer of neo^r by growth in media containing G418, and assayed for expression of the muscle phenotype. Initially, the secondary microcell hybrids were scored for the ability to form myotubes under differentiation inducing conditions by visual inspection.

From this analysis, it was obvious that C2 (Rh18)-11 transmitted the non-muscle phenotype with the neo^r marker, as five out of six clones, named the C2Rh1811 family, did not display myotube formation. Figure 1-2 shows a photomicrograph of the secondary microcell hybrid C2Rh1811-6, compared to parental C2C12 cells under differentiation inducing conditions. The non-differentiating phenotype of the secondary microcell hybrids was confirmed by northern blot hybridizations, and is similar to C2 (Rh18)-11. A similar *in situ* hybridization analysis of the secondary microcell hybrids from C2 (Rh18)-11 revealed that only a large rearranged human chromosome was transferred. In contrast, all of microcell hybrid clones, at least ten clones each, generated from C2(Rh18)-3 and C2(Rh18)-D displayed extensive cell fusion and myotube formation. In addition, northern blot is hybridizations showed that these secondary clones expressed MLC and MHC mRNAs at parental C2C12 levels. Therefore, it is unlikely that

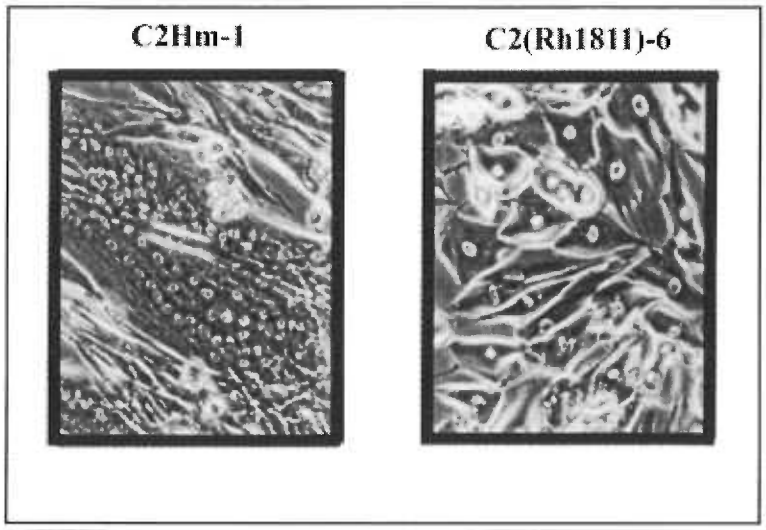

Figure 1-2. Lack of myotube formation in C2 (Rh1811)-6. Phase-contrast micrographs of myotube formation of mouse myoblast C2C12 cells and the secondary microcell hybrid C2 (Rh1811)-6 are shown. Cells were grown to confluence and then placed in differentiation medium for 48 hours. The multinucleated syncitium is representative of myotube formation.

the neo^r insertions in these other primary microcell hybrids are linked to the inhibitory loci. Characterization of the loci involved in inhibition of myogenesis in C2 (Rh18)-3 and C2 (Rh18)-D awaits the isolation of additional primary clones with different neo^r insertions.

In order to characterize the large rearranged chromosome present in C2 (Rh18)-11 and its secondary microcell hybrid derivatives, DNA marker analysis with genes and unique sequences known to map to specific chromosomes was performed. The marker analysis (see Table 1-1) along with the G-banding studies confirmed that chromosome 14 sequences were present on the large chromosome originating from the Rh18 cell line. The altered chromosome was classified as a derivative 14 chromosome containing a large homogeneous staining region (HSR) near the center of the chromosome. Homogeneous

staining regions are often associated with tumors and cell line derived from tumor specimens. Since the derivative 14 chromosome was large, a third round of microcell hybrids was generated in C2C12 using C2 (Rh1811)-6 as the donor cell line was performed. It has been shown that microcell fusion can be used to obtain hybrids containing a variety of sub-chromosomal fragments (Leach et al., 1989). The co-transfer frequency can be used to group specific chromosomal markers into nearest neighbor assemblages. We therefore planned to generate fragment containing hybrids in order to map and identify the region responsible for inhibition of muscle differentiation. Twenty-one tertiary microcell hybrids, the C2hi (R1811) family, were selected for analysis. Approximately fifty percent of these clones failed to differentiate into muscle based on visual inspection.

The initial marker analysis consisted of chromosome 14 specific probes. Both PCR and southern hybridization were employed in screening a microcell hybrid panel consisting of primary, secondary, and tertiary microcell hybrid clones. Southern hybridization experiments established the presence in single copy number of MAX, FOS, and TGFβ3 indicating that chromosome 14 sequences were present. We chose to utilize PCR technology on order to rapidly identify regions of DNA both present and absent from the derivative 14 chromosome. The PCR analysis used primers specific for genes previously mapped to chromosome 14. In addition, highly informative chromosome 14 specific microsatellite markers were employed. Recently, the number of available microsatellite markers has increased allowing for the construction high-density maps. Maps based on these markers have contributed greatly to primary mapping of a number

of diseases (Litt and Luty, 1989; Wolf M et al., 1997). Unfortunately, our use of chromosome 14 markers failed to yield informative information with regard to the

Human Chromosome 14 PCR Marker Analysis

				PCR Primer Sets											
Cell Line	Phenotype	PI	NP	D14S274	D14S63	D14S255	D14S254	D14S277	D14S66	D14S67	D14S72	D14S78	D14S80	D14S34	TGFB3	FOS
C2C12	Muscle	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
H.Myoblast	Muscle	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Rh18	NonMuscle	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
HDM-5	Muscle	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+
C2Rh18-3	NonMuscle	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C2Rh18-D	NonMuscle	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C2Rh11-11	NonMuscle	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C2(Rh1811)-1	NonMuscle	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C2(Rh1811)-2	NonMuscle	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+
C2(Rh1811)-3	NonMuscle	-	+	+	+	-	-	-	+	-	+	-	+	-	+	+
C2(Rh1811)-4	Muscle	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
C2(Rh1811)-5	NonMuscle	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+
C2(Rh1811)-6	NonMuscle	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C2hiR1811-A	NonMuscle	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+
C2hiR1811-B	NonMuscle	-	+	+	+	+	+	+	+	+	-	-	+	+	+	+
C2hiR1811-C	NonMuscle	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C2hiR1811-D	Muscle	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C2hiR1811-E	Muscle	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-
C2hiR1811-F	Muscle	-	-	-	-	-	-	-	+	+	-	+	-	-	-	-
C2hiR1811-G	Muscle	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
C2hiR1811-I	NonMuscle	-	+	+	+	+	+	+	+	+	-	-	+	+	+	+
C2hiR1811-J	NonMuscle	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C2hiR1811-K	NonMuscle	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C2hiR1811-L	Muscle	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
C2hiR1811-M	NonMuscle	-	+	+	+	+	-	+	+	+	+	+	+	+	-	+
C2hiR1811-N	Muscle	-	+	+	+	+	-	+	+	-	+	-	+	-	-	+
C2hiR1811-O	NonMuscle	-	+	+	+	+	+	+	+	+	+	-	+	+	-	+
C2hiR1811-P	Muscle	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C2hiR1811-Q	Muscle	-	-	-	-	-	-	-	+	+	-	-	+	-	-	+
C2hiR1811-R	Muscle	-	+	+	+	+	+	+	+	+	-	+	+	+	+	-
C2hiR1811-S	NonMuscle	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+
C2hiR1811-T	NonMuscle	-	+	+	+	+	-	+	+	-	+	-	+	-	-	+
C2hiR1811-U	Muscle	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
C2hiR1811-W	Muscle	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-

Table 1-1. Human chromosome 14 PCR marker analysis of microcell hybrids. All PCR reactions were carried out as described in Methods and analysis by agarose gel electrophoresis. Hybrids were scored for the presence (+) or absence (-) of the specific marker via ethidium bromide staining.

segments of DNA that segregated with the non-muscle phenotype. Table 1-1 illustrates the data generated from our chromosome 14 PCR marker analysis.

One explanation for the failure of DNA marker analysis to detect regions of chromosome 14 that segregated with the non-muscle phenotype, would be that the intervals between each marker are to great to allow for a thorough representation of all regions of chromosome 14. Therefore, in order to identify the chromosomal regions of interest, we decided to screen two YAC libraries. One of the YAC libraries, which was obtained from the Litt laboratory, and consisted of a continuous array of YAC clones encompassing the entire human 14 chromosome. The chromosome 14 library was

screened by hybridization with inter-Alu probes that mapped to the non-muscle chromosome (See below). No hybridization was detected with four inter-Alu probes used to screen the chromosome 14 YAC library, although hybridization with a FOS probe isolated specific YAC clones. We screened the second library, consisting of the entire human genome, using PCR primers designed from sequences contained within inter-Alu clone H531-3. Although, screening with PCR resulted in the identification of specific YAC clones, none of the corresponding YAC clones were localized to human chromosome 14. These findings suggest the chromosome 14 detected in the secondary microcell hybrids may be the result of a chromosomal translocation between chromosome 14 and another human chromosome.

Amplified DNA Segregates with the Non-Muscle Phenotype

Genomic instability is a central characteristic of cancer cells. A variety of genetic lesions are found in tumors, including rearrangement, gene amplification, point mutation, deletion, and acquisition of viral genomes. Gene amplification is one of the most common types of genetic changes occurring in human neoplasia (Garson et al., 1986; Garvin et al., 1986; Litt and Luty, 1989; Mitani et al., 1986; Roberts et al., 1989; Wolf M et al., 1997). Likewise, chromosomal translocations, which often result in gene fusion products, are prevalent in many forms of cancer (Bishop, 1987; Jin et al., 1993). The presence of the HSR within the derivative 14 chromosome is suggestive of gene amplification, since HSR regions have been shown to contain amplified DNA in tumor cell lines. To determine whether only chromosome 14 sequences are present on this

chromosome we utilized fluorescent in situ hybridization (FISH) with a chromosome 14-specific "paint" as probe. Figure 1-3b shows that chromosome 14 sequences are present in the centromeric and telomeric regions of this chromosome, but are excluded from the HSR. Therefore, the HSR is likely to contain sequences from elsewhere in the genome.

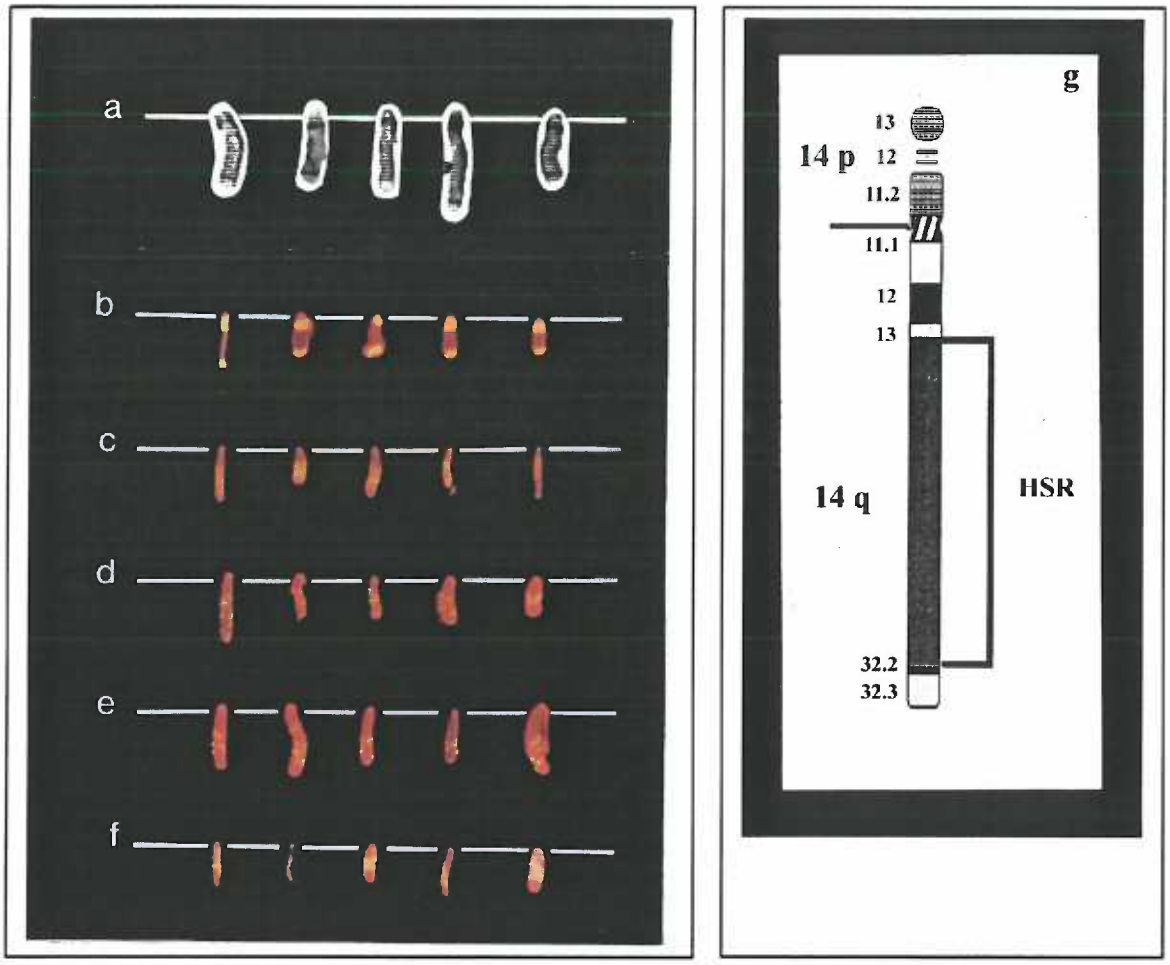

Figure 1-3. Cytogenetic characterization of the derivative chromosome 14. (a) G-banded preparations of the derivative chromosome 14 present in the secondary microcell hybrid C2 (Rh1811)-6. (b) FISH with the derivative 14 probed with a chromosome 14-specific paint (Oncor). (c) FISH with the derivative 14 chromosome probed with chromosome 12-specific paint (Oncor). (d to f) FISH with the derivative chromosome 14 probed with human cosmid clones Cospil-4, Cos60, and Cos85, respectively. These human cosmid clones detect single-copy DNA in normal human DNA and amplified DNA in Rh18 and secondary microcell hybrids carrying the derivative 14 chromosome as assayed by southern analysis (Figure 1-7). (g) Schematic diagram of the derivative chromosome 14, showing the location of the HSR.

To identify the chromosomal origin of the HSR, we isolated human-specific probes from the derivative 14.

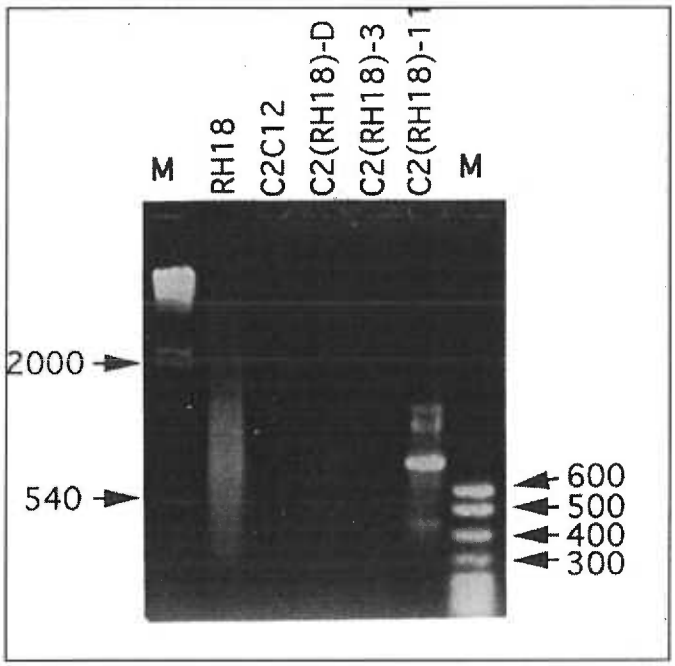

Figure 1-4. Inter-Alu PCR products from the primary non-muscle microcell hybrids. PCR was carried out on 1 μg of genomic DNA with a single Alu primer 517. The products were separated by electrophoresis through a 1% agarose gel and visualized by ethidium bromide staining.

Inter-Alu PCR was developed to isolate human-specific sequences directly from somatic cell hybrids using PCR with primers directed at the human Alu repeat element (Ludecke et al., 1989; Nelson et al., 1991; Nelson et al., 1989) . Figure 1-4 shows the inter-Alu PCR products generated from DNA isolated from the three primary microcell hybrids C2 (Rh18)-3, C2 (Rh18)-11, and C2 (Rh18)-D compared to Rh18 and C2C12 DNA. The smear of PCR products from Rh18 DNA is expected and is derived from amplification products from all chromosomes. Inter-Alu PCR products are easily detected from C2 (Rh18)-11 DNA, but not from C2 (Rh18)-3 or C2 (Rh18)-D DNA even though they retain two to three human chromosomes. The intensity of the ethidium bromide stained bands of the inter-Alu PCR products from C2 (Rh18)-11, even after relatively low cycle number, suggests that the derivative 14 contains amplified DNA. Furthermore, increasing the number of cycles

during the PCR reaction results in amplification of inter-Alu PCR products from C2 (Rh18)-3 and C2 (Rh18)-D (Thayer, unpublished).

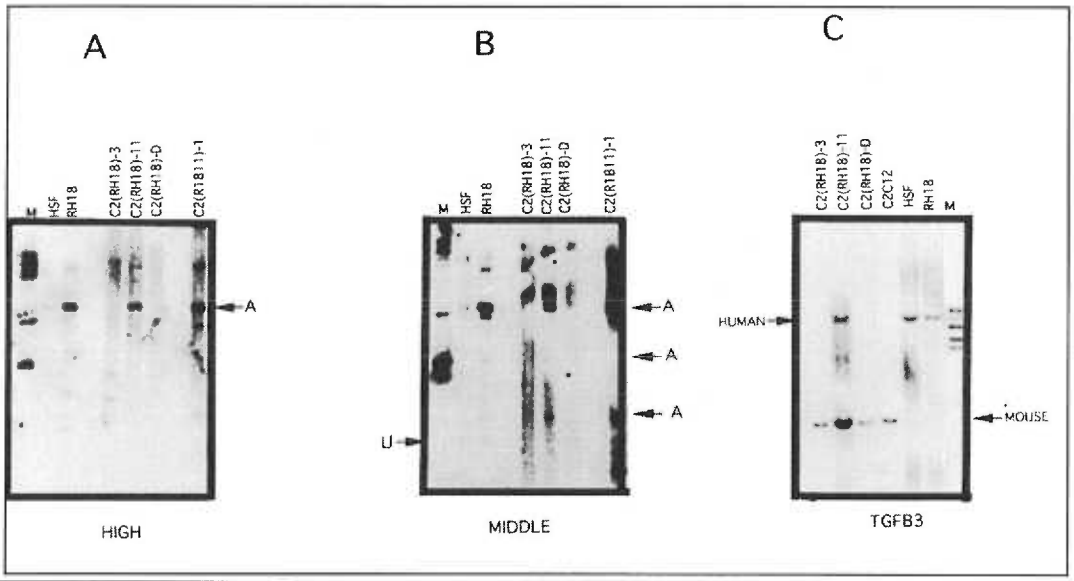

Figure 1-5. Inter-Alu PCR products detect amplified DNA sequences. Southern blot hybridizations with 10μg of genomic DNA are shown. Hybridizations were carried out in hybridization buffer overnight over-night at 65◦C. Probes were random prime labeled using [^{32}P]dCTP. Arrow A, amplified DNA sequences detected by the probe; Arrow U, unamplified DNA sequences detected by the probe. (A) Hybridization with pooled inter-Alu PCR products representing high-molecular weight products (1000 to 2000 bp) using primer 517. (B) Hybridization with pooled inter-Alu PCR products representing middle-molecular weight products (500 to 1000 bp) generated with primer 517. (C) Hybridization with TGFβ-3 as a probe. TGFβ-3 detects both mouse and human sequences and is not amplified in Rh18 or microcell hybrid C2 (Rh18)-11. HSF is Human Skin Fibroblast and molecular marker.lane M,

To determine whether the inter-Alu PCR products obtained from C2 (Rh18)-11 are derived from amplified DNA, we performed Southern blot hybridizations under conditions that suppress hybridization of Alu repeats (Budowle and Baechtel, 1990) . Inter-Alu PCR products were size fractionated on an agarose gel and isolated as pools of fragments representing high (1000-2000bp), middle (500-1000bp) and low (200-500bp) molecular weight fragments. Figure 1-5 (panels A and B) shows that both the high and middle molecular weight probes detect amplified bands in Rh18, C2 (Rh18)-11, and the secondary microcell hybrid C2Rh1811-1, when compared to primary human skin

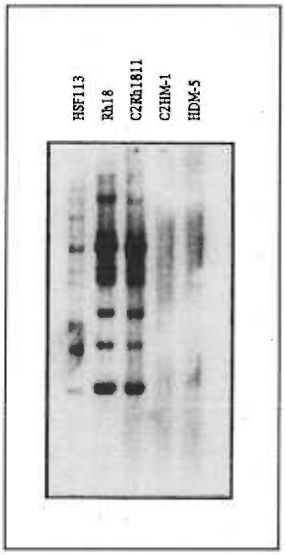

Figure 1-6. Inter-Alu products detect amplified DNA sequences. Southern blot hybridization with pooled inter-Alu products generated with primers 3' and 5'. HDM-5, somatic cell hybrid carrying a single human chromosome 14.

fibroblast (HSF) DNA. In addition, the middle probe detects an unamplified band, which serves as an internal control for both quantity and quality of the HSF DNA. By utilizing different Alu primers and varying the PCR conditions used to amplify the products, we have been able to isolate twenty eight different inter Alu PCR products that detect amplified DNA in Rh18 and the derivative 14 microcell hybrids. In order to facilitate in the characterization of the inter-Alu products, the initial PCR products were cloned into the plasmid pBS-SK vector (Stratagene). We have mapped the inter-Alu PCR products, which detect amplified DNA, to normal chromosome 12 by utilizing Southern blot hybridizations to a somatic cell hybrid mapping panel. In addition, chromosome 14-specific probes (TGFB3, MAX and FOS) do not detect amplified DNA. Figure 1-5C shows that TGFB3 is not amplified in Rh18 DNA, and is present in the microcell hybrid C2 (Rh18)-11 at single copy. Similar results were obtained with MAX and FOS probes, as mentioned above. Furthermore, utilizing FISH and a chromosome 12-specific "paint", we show that the DNA in the derivative 14 HSR originated from chromosome 12 (Figure 1-3C). Therefore, it seems likely that the derivative 14 consists of chromosome 14 sequences present at single copy, and chromosome 12 sequences that have been amplified.

A recent study demonstrated that transfer of single human chromosomes into 10T1/2 fibroblasts resulted in the inhibition of MyoD to activate the muscle

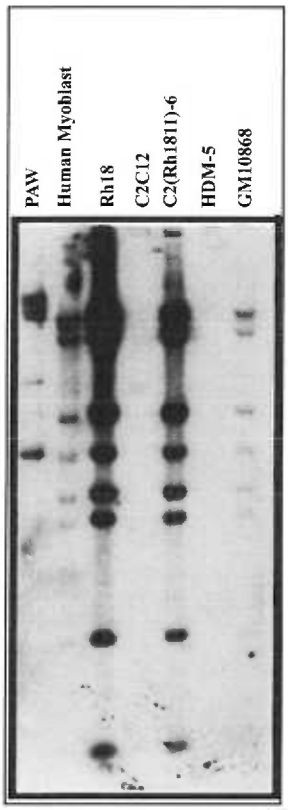

Figure 1-7. Cosmid clone Cos-60 is amplified in Rh18 and C2(Rh1811)-6. Southern blot hybridization with Cos-60 cosmid clone as a probe is shown. Ten μg of genomic DNA was extracted from human myoblasts, Rh18, C2C12, C2(Rh1811)-6, HDM-5, and GM10868. HDM-5 and GM10868 are somatic cell hybrids carried single copies of human chromosome 14 and 12 respectively.

differentiation program in these cells (Woloshin et al., 1995). Therefore in order to determine if normal chromosome 12 and 14 are capable of inhibiting muscle differentiation in C2C12 cells microcell hybridizations were performed using somatic cell hybrids carrying single human chromosomes 12 and 14 into donor C2C12 cells. Transfer of chromosome 12 into C2C12 resulted in hybrids that differentiated into myotubes and produced MHC as detected by immunohistochemistry at levels equal to those found in C2C12 cells (Fiddler, unpublished). Likewise C2C12 hybrids containing human chromosome 14 differentiated into myotubes and expressed muscle specific markers at similar levels to those found in C2C12 cells (Fiddler, unpublished). These results indicate that transfer of neither chromosome 12 nor 14 have the ability to inhibit muscle differentiation in skeletal myoblasts. Given that the derivative 14 chromosome contains DNA from chromosomes 12 and 14, with sequences from 14 being found at single copy concentrations, it seems likely that amplification of chromosome 12 sequences is responsible for the non-muscle phenotype.

To obtain a larger number of probes to the DNA amplification, a cosmid library was constructed from the secondary microcell hybrid C2(Rh1811)-6. The cosmid library was screened with labeled human DNA to identify Alu containing clones.

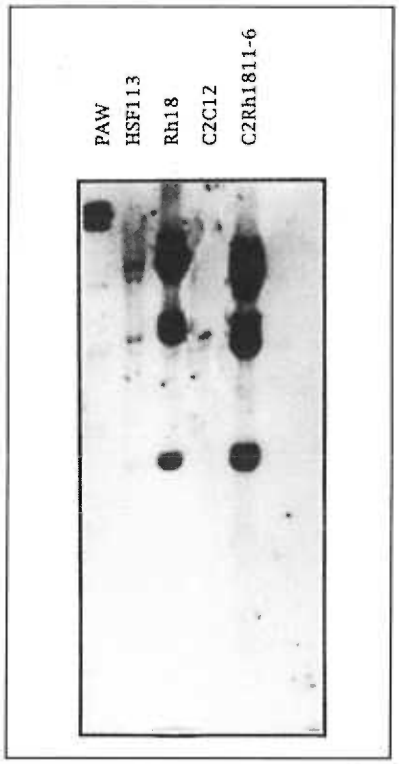

Figure 1-8. Mdm2 is amplified in Rh18 and in C2 (Rh1811)-6 microcell hybrid. Southern blot hybridization with MDM2 cDNA as a probe is shown. Ten micrograms of genomic DNA was extracted from primary HSFs (HSF113), Rh18, C2C12, and C2 (Rh1811)-6. PAW, molecular weight marker.

Approximately 3% of the total clones represented within the C2(Rh1811)-6 cosmid library hybridize with the human-specific probe; the remaining 97% presumably are derived from C2C12 DNA and represent mouse sequences. Twenty human-specific cosmids were isolated and used as probes for Southern blot hybridizations. Ten of twenty human-specific cosmids detected amplified DNA present on the derivative 14 (Fiddler, unpublished). Figure 1-7 shows cosmid DNA hybridized to southern blot. Thus, 1-2% of the cosmid clones in this library are derived from the DNA amplification. Figure 1-3d-f shows FISH of three cosmid clones hybridizing to the derivative 14 HSR. These three cosmids were also localized by FISH to normal 46XY chromosome spreads and found to map to 12p13-15.

Amplification of cellular proto-oncogenes have been described in a number of different tumor types, including rhabdomyosarcomas (Garson et al., 1986; Garvin et al., 1986; Mitani et al., 1986; Roberts et al., 1989). Because the HSR present on the derivative 14 contains chromosome 12 sequences, and a gene (GLI) known to reside on chromosome 12, located at 12q13-14.3 (Kinzler et al., 1987) has been shown to be amplified in 1 out of 13 rhabdomyosarcomas (Roberts et al., 1989) , we next determined whether genes located on chromosome 12 were present on the derivative 14. I have

determined that GLI, SAS, CHOP, LALB-A, RARG1, VDR, COL2A, CDK4 and MDM2 are present on the derivative 14 and show varying levels of amplification. Figure 1-8 shows a representative Southern blot using MDM2 as probe. In addition, the following chromosome 12 genes do not reside on the derivative 14: MYF-5, TEL, RAP1B, CDK-2, CCND2, SP1, HOXC5, WNT1, A2MR, ATF-1, BTG, ASCL1, PRPH and ERBB-3. To further characterize the chromosome 12 sequences present within the derivative 14, PCR with chromosome 12 specific microsatellite markers was employed (Table 1-2).

Human Chromosome 12 Marker Analysis

		PCR Primer Sets								
Cell Line	Phenotype	D12S85	D12S82	D12S84	D12S106	D12S101	D12S81	D12S87	D12S58	D12S345
C2C12	Muscle	-	-	-	-	-	-	-	-	-
H.Myoblast	Muscle	+	+	+	+	+	+	+	+	+
Rh18	NonMuscle	+	+	+	+	+	+	+	+	+
GM10868	Muscle	+	+	+	+	+	+	+	+	+
C2Rh18-3	NonMuscle	-	-	-	-	-	-	-	-	-
C2Rh18-D	NonMuscle	-	-	-	-	-	-	-	-	-
C2Rh11-11	NonMuscle	-	+	+	+	+	+	-	+	+
C2(Rh1811)-1	NonMuscle	-	-	-	+	+	+	-	+	-
C2(Rh1811)-2	NonMuscle	-	-	-	+	-	-	-	+	-
C2(Rh1811)-3	NonMuscle	-	-	-	+	+	+	-	+	-
C2(Rh1811)-4	Muscle	-	-	-	+	-	+	-	+	-
C2(Rh1811)-5	NonMuscle	-	-	-	+	+	+	-	+	-
C2(Rh1811)-6	NonMuscle	-	-	-	+	-	+	-	+	-

		PCR Primer Sets					
Cell Line	Phenotype	D12S64	D12S92	D12S95	D12S96	D12S313	D12S75
C2C12	Muscle	-	-	-	-	-	-
H.Myoblast	Muscle	+	+	+	+	+	+
Rh18	NonMuscle	+	+	+	+	+	+
GM10868	Muscle	+	+	+	+	+	+
C2Rh18-3	NonMuscle	-	-	-	-	-	-
C2Rh18-D	NonMuscle	-	-	-	-	-	-
C2Rh11-11	NonMuscle	-	+	+	+	+	-
C2(Rh1811)-1	NonMuscle	-	-	+	+	-	-
C2(Rh1811)-2	NonMuscle	-	-	+	+	-	-
C2(Rh1811)-3	NonMuscle	-	+	+	+	-	-
C2(Rh1811)-4	Muscle	-	-	+	+	-	-
C2(Rh1811)-5	NonMuscle	-	+	+	+	-	-
C2(Rh1811)-6	NonMuscle	-	+	+	+	-	-

Table 1-2. Human chromosome 12 PCR marker analysis of microcell hybrids. All PCR reactions were carried out as described in Methods and analysis by agarose gel electrophoresis. Hybrids were scored for the presence (+) or absence (-) of the specific marker via ethidium bromide staining.

Concurrent with our molecular characterization of the HSR region, inter-Alu and cosmid clones were further classified to determine whether these probes contained

transcribed sequences. Northern analysis was carried out on those clones, which detected amplified DNA from the HSR region. In the human genome Alu DNA constitutes the dominant family of short repetitive sequences. Each of these sequences is about 300 base pairs (bp) long (Britten et al., 1988). Alu sequences are efficiently transcribed, accounting for a significant fraction of hnRNA (Britten et al., 1988; Filatov et al., 1991). In the course of processing, the majority of Alu transcripts are degraded. The small portion of Alu containing transcripts share homology with the RNA component of the signal recognition particle (7SRNA). The presence of Alu elements in the intervening sequences and untranslated regions of a number of structural genes also contributes to the observed expression of the Alu family (Onno et al., 1992; Yeo et al., 1994). Alu sequences are dispersed through out the genome at approximately 4000 bp intervals. Since all of the inter-Alu PCR products contain Alu sequences, and with the average cosmid size being greater than 25 kilobase pairs in length (Kb) suppression of the Alu element was necessary in order to identify non Alu transcripts. We therefore, performed these Northern blot hybridizations under conditions that suppress hybridization of Alu repeats (Budowle and Baechtel, 1990). Two of the initial cosmid clones and one inter-Alu product detected expressed sequences on northern hybridization.

Clones were selected from a cDNA library prepared with cytoplasmic poly (A) RNA of rhabdomyosarcoma Rh18 cell line. The Rh18 cDNA library was constructed in the lambda Zap system (Stratagene) and consisted of seven million independent clones. Clones were selected by southern hybridization using conditions that suppress hybridization to Alu repeats. Two cDNA clones were isolated, cH5313-4A from an inter-Alu library screen, and cCos60-3 from a cosmid clone used to screen the Rh18 cDNA

library. The cDNA clones were characterized by restriction analysis through agarose gel electrophoreses. cH5313-4A was shown to be approximately 3.5 KB in length, while cCos60-3 was demonstrated to be approximately 1400 bp in length. Both cDNA clones were further analyzed by southern hybridization and shown to detect amplified DNA in cell lines containing the derivative 14 chromosome. This result was exactly what we predicted based on the fact that probes used to isolate the cDNA clones were themselves amplified. Figure 1-10 shows southern hybridizations using cH5313-4A as a probe. Northern hybridization experiments were carried out to determine the full length of the isolated cDNA clones. cCos60-3 hybridized to a transcript of approximately 1400 bp in length indicating that this cDNA is a full-length clone. The cH5313-4A clone detected a 5 Kb transcript suggesting it as being a partial cDNA (Figure 1-9).

Plasmid inserts were sequenced using the dideoxy chain termination method. Analysis of the sequence data in cCos60-3 revealed that this cDNA is a member of the ubiquitin containing gene family. Because a portion of the generated sequence failed to align with any known gene product it seems likely that cCos60-3 is a ubiquitin fusion gene. Comparative northern analysis between the rhabdomyosarcoma cell lines and normal human fibroblasts detected overexpression of cCos60-3 RNA in all rhabdomyosarcoma cell lines at levels five to six times that of normal cells (Fiddler, unpublished). Our initial experiments grouped the rhabdomyosarcoma cell lines into two categories, dominant and recessive, with regard to their failure to display the muscle phenotype. Because cCos60-3 appears to be over expressed in all rhabdomyosarcoma cell lines tested it seems unlikely that cCos60-3 was responsible for the dominant non-muscle phenotype seen in the Rh18 cell line. Furthermore, expression of cCos60-3 appeared to

be equivalent levels in microcell hybrids that differentiated in to muscle and those that fail to form myotubes.

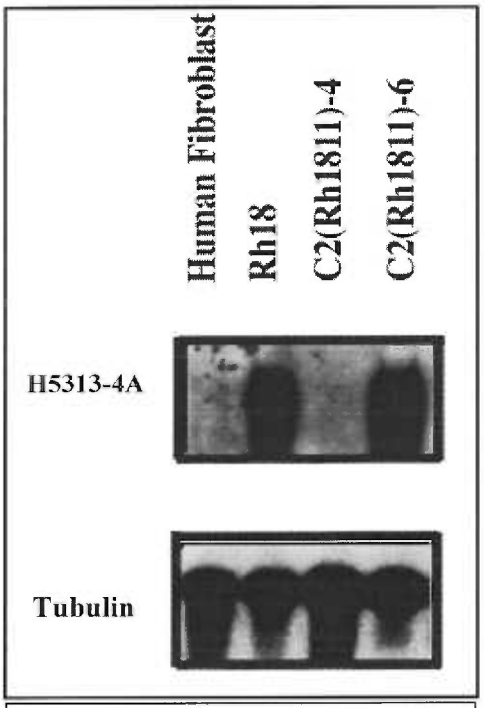

Figure 1-9 Over-expression of clone cH5313-4A in Rh18 and secondary microcell hybrid C2 (Rh1811)-6. Northern blot analysis to 5 μg cytoplasmic RNA. The probes are full-length cDNA cH5313-4A and α-tubulin.

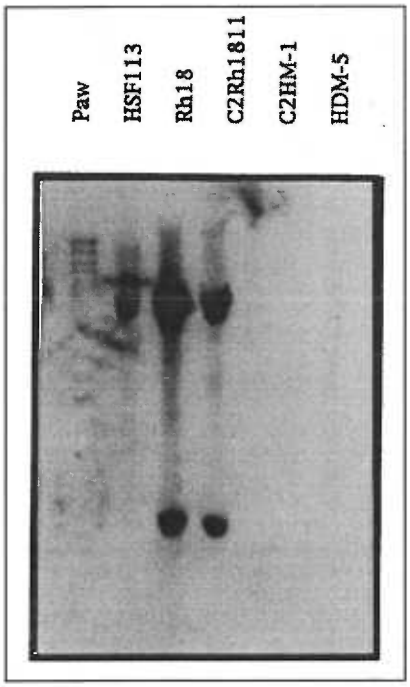

Figure 1-10. Amplification of cDNA clone cH5313-4A in Rh18 and primary microcell hybrid C2 (Rh18)-11. Southern blot hybridization with cH5313-4A as a probe. Ten micrograms of genomic DNA from human skin fibroblast (HSF113), mouse myoblast (C2HM-1), human chromosome 14 bearing somatic cell hybrid (HDM-5), and molecular marker (PAW).

MDM2 Gene Amplification and Overexpression

Sequence analysis of the cH5313-4A clone revealed that this cDNA contained intronic sequences specific for the MDM2 oncogene. Apparently, cH5313-4A is the result of an inappropriately spliced or unspliced transcript. This was extremely interesting because MDM2 was shown to be present and amplified on the derivative 14

chromosome. To further characterize MDM2 we performed southern hybridizations using the full length MDM2 cDNA as a probe. MDM2 was found to be present, at single copy amounts, in all of the rhabdomyosarcoma cell lines while only being amplified in the Rh18 cell line. Furthermore, hybridization of the MDM2 cDNA to the microcell hybrid panel resulted in the detection of amplified MDM2 sequences in all microcell hybrid that failed to differentiate into muscle. The microcell hybrids that effectively demonstrate myotube formation detected no human MDM2 sequences. Genomic amplification of MDM2 is often associated with overexpression of the MDM2 gene (Fiddler et al., 1996; Ladanyi et al., 1993; Reifenberger et al., 1994). To determine whether MDM2 is over expressed, we performed northern hybridization using the human full length MDM2 cDNA as a probe (Figure 1-11). We found MDM2 to be over-expressed only in Rh18 and those microcell hybrids that displayed failure of the muscle phenotype. Taken together

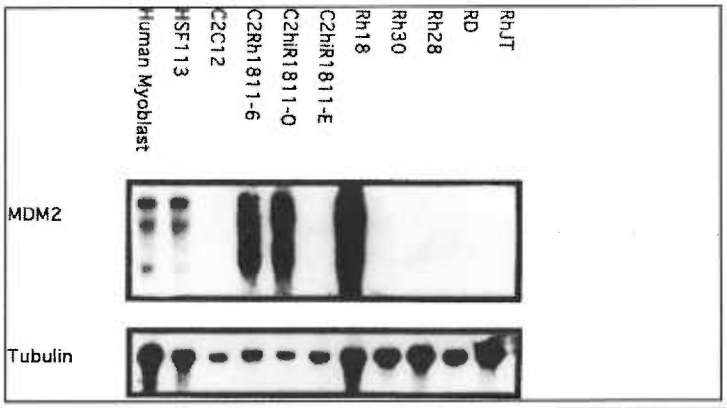

Figure 1-11. MDM2 is overexpressed in Rh18 and the derivative 14 chromosome microcell hybrids. Northern blot hybridization to 5$_{\mu}$g of cytoplasmic RNA extracted from primary human myoblasts; primary HSFs (HSF113); C2C12; the secondary microcell hybrids C2 (Rh1811)-6, C2hiR1811-O, and C2hiR1811-E, Rh18; and the recessive rhabdomyosarcomas Rh30, Rh28, RD, and RhJT are shown. The probes were a full-length MDM2 cDNA or $_{\alpha}$-tubulin. MDM2 transcripts are detected from all cell lines following longer exposure (not shown).

this data is highly suggestive of MDM2 being involved with the non-muscle phenotype.

In order to further characterize those genes localized on the derivative 14, northern analysis was performed. CDK4 was found to be over expressed in all the rhabdomyosarcoma cell lines as well as microcell hybrids that failed differentiate into muscle. Alternatively, CHOP, GLI, VDR and MYF-5 hybridization detected transcript levels that appeared consistent between all rhabdomyosarcomas and microcell hybrids. The SAS gene, which is amplified in Rh18, Rh28, and Rh30, was also found to be overexpressed in non-muscle forming microcell hybrids. Recent evidence reported SAS as a member of a transmembrane protein family thought to be involved in growth-related cellular processes. Since both CDK4 and SAS overexpression was detected in non-muscle forming microcell hybrids, both these genes also became candidates for inhibition of the muscle phenotype. With both genes being overexpressed in other rhabdomyosarcomas in addition to Rh18, it appears that these genes may have a more generalized role in tumorgenesis than that specifically associated with failure to differentiate into mature muscle fibers.

MDM2 Inhibits Muscle-Specific Gene Transcription

To determine whether the failure to differentiate observed in C2 (Rh18)-11 and its secondary microcell hybrids was due to an inability of MyoD to activate transcription, as well as to establish a simple assay system to test candidate genes, we assayed muscle-specific gene expression by transient transfection. The muscle-specific reporter constructs consisted of the CAT gene driven by either the muscle creatine kinase

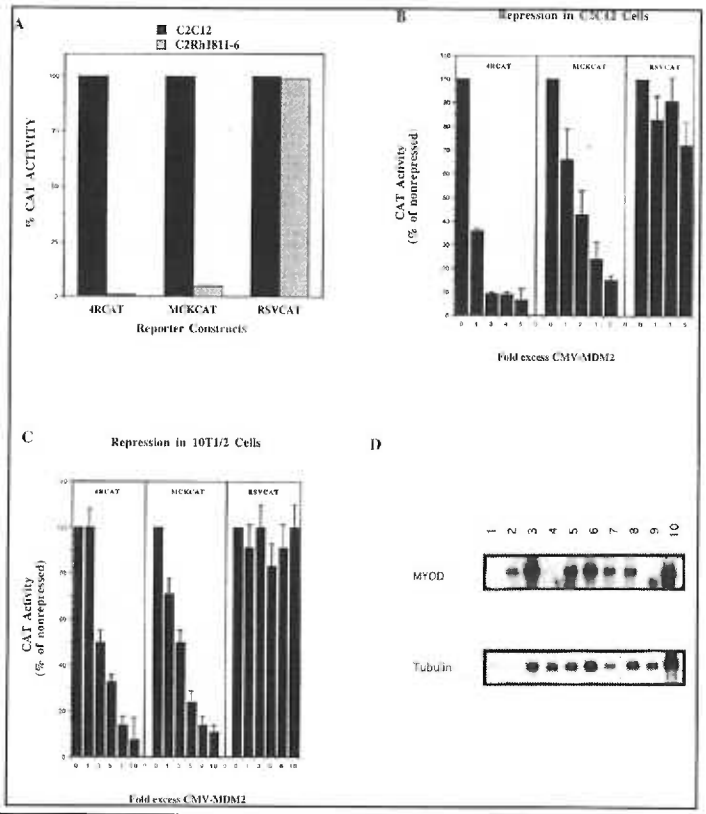

Figure 1-12. MDM2 represses MyoD-dependent transcription. (A) Parental C2C12 and microcell hybrid C2 (Rh1811)-6 were transfected with the muscle specific reporter constructs MCKCAT and 4RCAT and the control plasmid RSVCAT. Cells were harvested at 72 hours, and CAT assays were performed. The CAT activity expressed C2C12 was set as 100%. (B) C2C12 cells were co-transfected with an MDM2 expression vector and the indicated reporter constructs. Increasing amounts of MDM2, designated as fold excesses with respect to the reporter construct, were added. Values represent the percentages of CAT activity obtained in the absence of MDM2. All transfections were kept at 6 μg of DNA by substituting an empty expression vector for the MDM2 expression vector. (C) 10T1/2 cells were transfected with 0.5 μg of a MyoD expression vector, 0.5 μg of the indicated reporter constructs, and increasing amounts of MDM2. MyoD induced CAT activity 60-fold on 4RCAT and 10 fold on MCKCAT compared to control assays lacking MyoD. (D) 10T1/2 cells were co-transfected as described for panel C, and RNA was extracted and processes for northern blot hybridization. The probes were full-length MyoD cDNA or α-tubulin. Lames: 1, 4RCAT; 2, 4RCAT and MyoD; 3, 4RCAT and MyoD; 4, MCKCAT; 5,MCKCAT and MyoD; 6, MCKCAT, MyoD, and 0.5 μg MDM2; 7, MCKCAT, MyoD, and 2.5 μg MDM2; 8, MCKCAT, MyoD, and 4.0 μg MDM2; 9, pSPORT2; 10, control C2C12 RNA (5.0 μg). All values representing transfection experiments are the averages from three separate experiments, each experiment containing duplicate dishes.

enhancer (MCKCAT) or four multimerized MyoD binding sites that were cloned upstream of the herpes simplex thymidine kinase promoter (4RtkCAT). Figure 1-12A

shows that the secondary microcell hybrid, C2Rh1811-6, contains significantly less MCKCAT and 4RtkCAT activity than parental C2C12 cells, while expression of RSVCAT was equivalent between the two cell lines. Therefore, the derivative 14 inhibits muscle differentiation and decreases muscle-specific gene expression.

To test the candidate genes for the ability to inhibit muscle differentiation, we have utilized the transient transfection system. To date, we have assayed CDK4, CHOP, cCos60-3, VDR, and MDM2 for the ability to inhibit expression of MCKCAT and 4RtkCAT. We have not observed any significant affect of CDK4, cCos60-3, VDR or CHOP in this assay, although large quantities of each gene mRNA was detected (Fiddler, unpublished). In contrast, transfection of C2C12 cells, with an MDM2 expression vector (Oliner et al., 1992) and various reporter constructs, demonstrates that MDM2 is capable of inhibiting expression of both MCKCAT and 4RtkCAT, but had little or no affect on RSVCAT (Figure 1-12B). To test whether MDM2 inhibition of muscle-specific transcription is due to inhibition of MyoD, a second series of transient transfections were performed. 10T1/2 cells lack the ability to initiate transcription from muscle-specific promoters in the absence of exogenously added MyoD. Therefore, we assayed MyoD dependent transactivation in 10T1/2 cells co-transfected with a MyoD expression vector and an MDM2 expression vector. Figure 12C shows that MDM2 represses MyoD dependent transactivation of the 4RtkCAT and the MCKCAT constructs. Figure 1-12D shows that co-transfection of 10T1/2 cells with increasing amounts of the MDM2 expression vector does not affect the level of MyoD RNA expressed from the MyoD expression vector. These results indicate that MDM2 is a potent inhibitor of MyoD function at the post-transcriptional level. Since myf-5, but not MyoD was expressed in

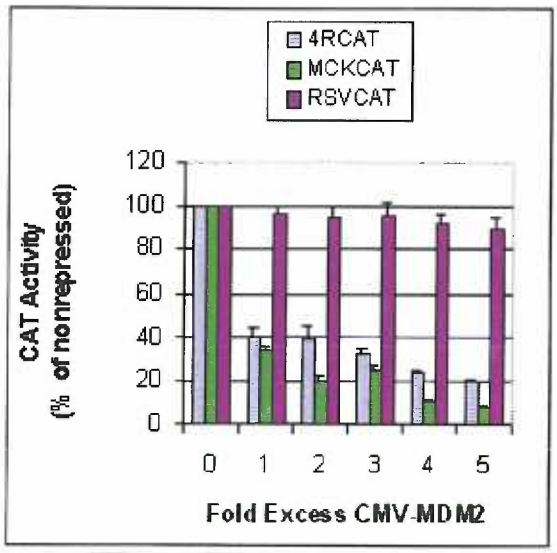

Figure 1-13. MDM2 represses myf-5 dependent transcription. 10T1/2 cells were transfected with 0.5 μg of myf-5 expression vector, 0.5 μg of the indicated reporter construct, and increasing amounts of MDM2. Myf-5 induced CAT activity 40 fold on 4RCAT and 15 fold on MCKCAT compared to control transfection lacking myf-5. Increasing amounts of MDM2, designated as fold excesses with respect to the reporter constructs added. All transfections were kept at a total of 6 μg of DNA by substituting an empty expression vector for the anti-sense MDM2 expression construct. The values are averages of at least three separate experiments, with each experiment containing duplicate plates.

rhabdomyosarcoma Rh18, we decided to determine if MDM2 could inhibit myf-5 transcriptional activity. Figure 1-13 shows co-transfection of 10T1/2 cells with myf-5 and increasing amounts on MDM2. Similar to MyoD, MDM2 can effectively inhibit myf-5 dependent transcription from the muscle specific reporter constructs 4RtkCAT and MCK-CAT.

One criterion for a candidate inhibitory gene is that it be expressed in the Rh18 cells, as well as in hybrids that retain the inhibitory locus. As mentioned above, we assayed expression of MDM2 in RNA isolated from Rh18 and the derivative chromosome 14 microcell hybrids by northern blot hybridization. Figure 1-11 shows that Rh18 and the secondary and tertiary microcell hybrids, C2Rh1811-6 and C2hiRh1811-O, express significantly more MDM2 mRNA than control primary human myoblasts and primary human skin fibroblasts. In contrast, the rhabdomyosarcomas Rh30, Rh28, RD, and RhJT, which display a recessive non-differentiating phenotype (Tapscott et al., 1993) , do not express high levels of MDM2 mRNA. Furthermore, the recessive rhabdomyosarcomas do not contain MDM2 DNA

amplification (Fiddler unpublished; Khatib et al., 1993). In addition, a tertiary microcell hybrid that retained myogenic potential, C2hiRh1811-E, does not retain MDM2 amplification and does not express high levels of MDM2 mRNA. Thus, overexpression of MDM2 correlates with the presence of the inhibitory locus in Rh18 and in the microcell hybrids that fail to differentiate into muscle.

Anti-sense MDM2 Rescues MyoD-Dependent Gene Transcription

To further investigate the ability of MDM2 to repress muscle differentiation, we introduced the CMV-MDM2 expression vector with a neo resistance gene stably into mouse myoblast C2C12 cells. Although the clones that carried the MDM2 construct formed myotubes on visible observation, the myotubes appeared smaller and less organized than those cells that where transfected with an empty CMV-expression vector. Following selection clones where evaluated for expression of muscle specific markers both through northern hybridization and histochemistry. Staining of MHC on a majority of the MDM2 stable clones revealed levels of MHC similar to those found in the control colonies. Likewise, expression of MyoD in the MDM2 stables was equal to that found in the controls. Interestingly, northern analysis revealed that none of the clones harboring the MDM2 expression construct expressed elevated levels of MDM2. It appears that transcription from the CMV promoter in some manner terminated, which would allow for the C2C12 cells to differentiate into myotubes following withdrawal serum from the culture media. Likewise, a recent study where MDM2 was stably transfected into SAOS-

2 resulted in transfected clone morphology indistinguishable from the parent cells (Fakharzadeh et al., 1991).

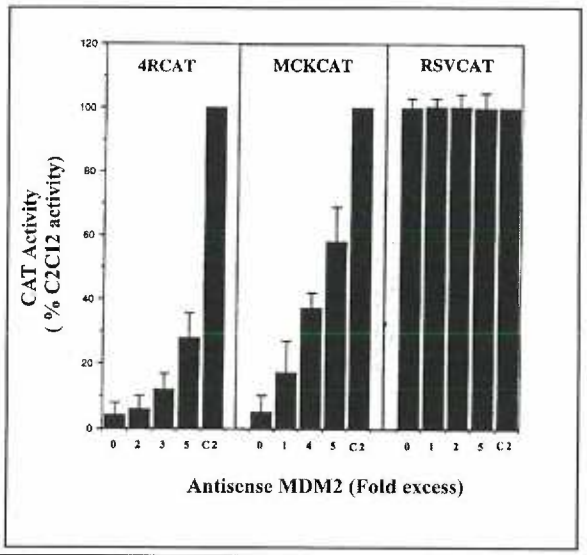

Figure 1-14. Expression of anti-sense MDM2 relieves repression of MyoD function. The microcell hybrid C2 (Rh1811)-6 was transfected with increasing amounts of anti-sense MDM2 expression vector and 1 μg of the indicated reporter construct. Values represent fold activation by anti-sense MDM2 compared with that with an empty expression vector. All transfections were kept at a total of 6 μg of DNA by substituting an empty expression vector for the anti-sense MDM2 expression construct. The values represent the averages from three separate experiments, with each experiment containing duplicate dishes.

To further test whether MDM2 is responsible for inhibition of MyoD function in our hybrid system, we tested whether expression of anti-sense MDM2 could restore muscle-specific gene expression in the derivative 14 microcell hybrids. The MDM2 cDNA was cloned in the anti-sense orientation under control of the CMV promoter. This construct was co-transfected with either MCKCAT, 4RtkCAT or RSVCAT into the secondary microcell hybrid C2Rh1811-6. Muscle-specific gene expression is restored when increasing amounts of anti-sense MDM2 are transfected into these cells (Figure 1-14). In addition, since the 4RCAT construct is activated by anti-sense MDM2, MyoD dependent transcription is restored by anti-sense MDM2. We conclude that amplification and overexpression of MDM2 inhibits MyoD function, resulting in a dominant non-differentiating phenotype.

Discussion

One obvious phenotype of tumor cells is a lack of terminal differentiation. Previously, we classified rhabdomyosarcoma cell lines as having either a recessive or a dominant non-differentiating phenotype (Tapscott et al., 1993) . To study the genetic basis of the dominant non-differentiating phenotype of Rh18 cells, we utilized microcell fusion to transfer chromosomes from Rh18 cells into the mouse myoblast cell line C2C12. Rh18 chromosomes were tagged with a selectable marker and transferred into C2C12 cells. Two different non-muscle phenotypes were obtained: one characterized by a complete lack of expression of MyoD and myogenin, and another, characterized by continued expression of MyoD and myogenin. Karyotypic analysis indicates that a derivative chromosome 14 is responsible for the MyoD and myogenin positive non-differentiating phenotype. The derivative chromosome 14 contains an amplification of chromosome 12 DNA sequences. MDM2 is amplified and overexpressed in Rh18 and in the non-differentiating microcell hybrids that retain the derivative 14. Forced expression of MDM2 in C2C12 cells, and in 10T1/2 cells transfected with a MyoD expression vector results in repression of MyoD dependent transcription. In addition, expression of anti-sense MDM2, in C2C12 hybrids containing the derivative 14, results in restoration of MyoD dependent transcriptional activity and therefore restoration of muscle-specific gene expression. We conclude that amplification and overexpression of MDM2 in rhabdomyosarcomas leads to inhibition of MyoD dependent transcription resulting in a dominant non-differentiating phenotype.

Gene amplifications are easily detected in tumors and transformed cell lines, while normal diploid cells lack detectable gene amplifications (Tlsty, 1990) . Gene amplifications are often responsible for drug resistance in cultured mammalian cells, and amplification of cellular oncogenes are often observed in tumors. In both cases, rare clones are selected because overexpression of genes through increased copy number is thought to confer a selective advantage (Stark et al., 1989) . Detailed mapping studies of amplified DNA indicates that regions far larger than the selected gene are always amplified. The size of amplification units can vary from a few hundred kilobases to as many as 10 megabases (Stark et al., 1989). In addition, other chromosomal abnormalities such as inversions, translocations, and deletions are often observed in conjunction with amplification. Given these observations, perhaps it is not surprising that the DNA amplification described here involves multiple genes encompassing a large region of genomic DNA, as well as involving two different chromosomes, 12 and 14.

Amplification of cellular proto-oncogenes has been described in a number of different tumor types, including rhabdomyosarcomas (Garson et al., 1986; Garvin et al., 1986; Mitani et al., 1986; Roberts et al., 1989). High frequency of MDM2 gene amplification has been observed in a variety of tumors, including bone and soft tissue sarcomas (Oliner et al., 1992) and gliomas (Reifenberger et al., 1994) . We have shown here that a number of genes from chromosome 12, including MDM2, GLI, and CDK4 are co-amplified in the rhabdomyosarcoma cell line Rh18. While it is not presently known how often MDM2 becomes amplified in primary rhabdomyosarcomas, at least one other rhabdomyosarcoma cell line retains ampified MDM2 (P. Houghton, personal communication). Regardless, MDM2 amplification is a common event in soft tissue

sarcomas (Khatib et al., 1993; Olson, 1992). and therefore represents a frequent genetic alteration in the generation of tumors. Interestingly, a similar DNA amplification is present in the recessive rhabdomyosarcoma cell line Rh30. In contrast to Rh18 however, Rh30 cells contain amplified GLI and CDK4 but not MDM2 (Khatib et al., 1993). In addition, C2C12 microcell hybrids that retain the Rh30 GLI and CDK4 amplified region continue to differentiate into muscle (Thayer, unpublished). This result is consistent with our observation that MDM2 amplification results in a dominant non-differentiating phenotype expressed in Rh18 cells, while Rh30 cells display a recessive phenotype and lack amplification of MDM2. Thus, amplification of GLI and CDK4 in Rh30 cells does not result in dominant inhibition of MyoD function. However, the functional consequences of GLI and CDK4 amplification on other tumor phenotypes, such as genomic instability or altered growth rate can be assayed using the system described here.

In addition to promoting tumor formation, anchorage independent growth, and cellular immortalization, expression of transforming oncogenes inhibits cellular differentiation in several different cell lineages. In muscle cells, expression of oncogenic tyrosine kinases (v-src and v-fps), growth factor receptors (v-erbB), nuclear oncogenes (v-myc, c-myc, v-erbA, and E1A), and the activated form of signal transducing G proteins (H-ras and N-ras) can inhibit terminal differentiation to varying extents (Falcone et al., 1985; Fiszman and Fuchs, 1975; Gossett et al., 1988; Holtzer et al., 1975; Olson et al., 1987; Payne et al., 1987; Schneider et al., 1987; Webster et al., 1988). I previously demonstrated that ras and fos prevent myogenesis by inhibiting expression of MyoD (Lassar et al., 1989). Identifying the specific pathway by which each of these oncogenes

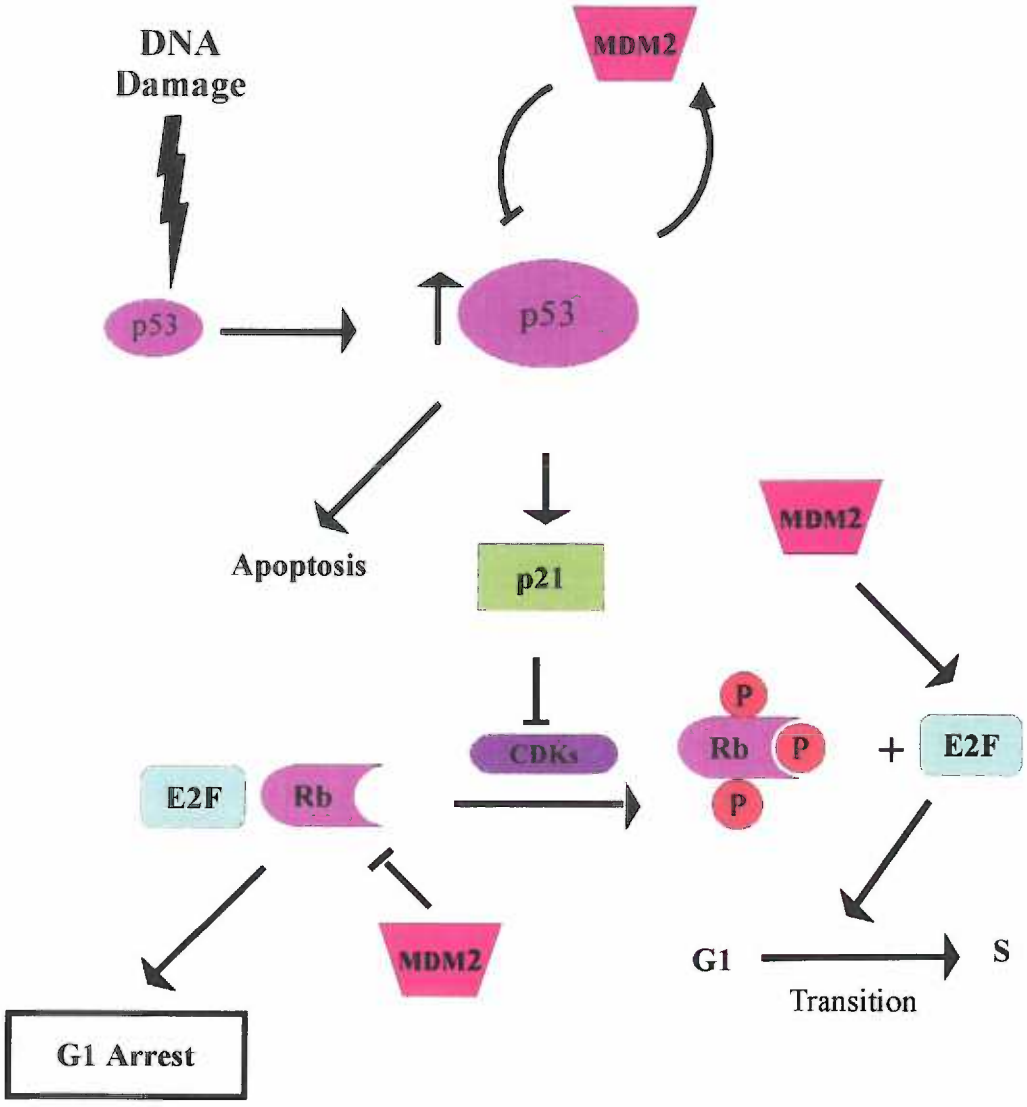

Figure 1-15. Model for MDM2 Promotion of Cell Cycle Progression.
MDM2 protein levels are increased through the transcriptional activity of p53. MDM2 completes the feedback loop by repressing p53 transcriptional activity through direct interaction with the p53 protein. In addition, MDM2 can negatively modulate Rb function through protein-protein interaction. The cell cycle promoting function of E2F is enhanced by binding to the MDM2 protein. An overabundance of MDM2, as seen with MDM2 gene amplification, can result in cellular proliferation through a variety of mechanisms.

and growth factors inhibits myogenesis may provide clues as to how MyoD integrates information coming from many aspects of cellular function.

The oncogenic properties of MDM2 have been postulated to result from direct interaction with a number of cell cycle regulatory proteins. MDM2 interacts directly with p53 (Oliner et al., 1992), and blocks p53 mediated transactivation by inhibiting the activation domain of p53 (Chen et al., 1993; Haines et al., 1994; Momand et al., 1992; Oliner et al., 1993; Zauberman et al., 1993). In addition, MDM2 has been shown to interact directly with pRB, resulting in stimulation of E2F/DP1 transcriptional activity and inhibition of pRB growth regulatory function (Xiao et al., 1995). Furthermore, MDM2 interacts with the activation domain of E2F1 resulting in stimulation of E2F1/DP1 transcriptional activity (Martin et al., 1995). Taken together, these results suggest that MDM2 not only relieves the proliferative block mediated by either p53 or pRB, but also promotes proliferation by stimulating the S-phase inducing transcriptional activity of E2F/DP1. Figure 1-15 illustrates potential mechanisms though which MDM2 can alter the cell cycle and thus inhibit differentiation.

Differentiating muscle cells fuse to form multinucleated myotubes and permanently withdraw from the cell cycle. This process is controlled by regulatory interactions involving MyoD family members and various cell cycle proteins (Lassar et al., 1994; Olson, 1992) . MyoD transactivation of muscle-specific genes requires wild-type pRB (Gu et al., 1993) or high levels of the pRB related protein p107 (Schneider et al., 1994) . In addition, transcriptional activation by MyoD has been shown to be inhibited by high levels of the G1-phase cyclin, Cyclin D1 (Rao et al., 1994; Skapek et al., 1995) . Furthermore, Cyclin D1 interacts with and promotes phosphorylation of pRB, resulting in stimulation of E2F/DP1 activity (Kato et al., 1993). While the mechanism by which MDM2 inhibits MyoD function remains unknown, it seems reasonable to

speculate that MDM2 either directly interferes with MyoD transactivation, as it does for p53, or that MDM2 inhibits MyoD indirectly by stimulating S-phase promoting factors such as E2F/DP1 and/or Cyclin D1, or by blocking pRB mediated MyoD transactivation. Regardless, the system described here, which combines somatic cell and molecular genetics, should allow for the characterization of the molecular mechanisms functioning in rhabdomyosarcomas to inhibit differentiation.

Chapter 2

MDM2 Repression of Muscle-Specific Gene Transcription is DNA Site Dependent

Background

Rhabdomyosarcoma

Rhabdomyosarcomas are one of the most common solid tumors of childhood, representing 4-8% of all malignant diseases under 15 years of age. Tumors arise *de novo* from skeletal muscle and, in some instances, demonstrate morphological characteristics of differentiated muscle tissue. Sarcomas have traditionally been classified as rhabdomyosarcomas based on morphology and expression of muscle structural genes, such as myosin heavy chain or desmin. Expression of the muscle determination gene MyoD has been shown to be the most sensitive marker for classifying sarcomas as rhabdomyosarcomas (Dias et al., 1990; Scrable et al., 1990). Rhabdomyosarcomas are grouped by histologic and cytogenetic criteria as either embryonal or alveolar rhabdomyosarcomas: a balanced translocation between chromosomes 2 and 13, t (2:13)(q35; q14), is associated with alveolar rhabdomyosarcomas (Barr et al., 1993). The PAX3 gene has been shown to be fused to a member of the forkhead gene family (FKHR) in the t (2:13) translocation (Barr et al., 1993; Shapiro et al., 1993). More recently, the PAX7 gene, located at 1p36, has been shown to be translocated to the FKHR gene as a 1:13 translocation (p36; q14) in some cases of alveolar rhabdomyosarcoma (Davis et al., 1994). Loss of heterozygosity on the short arm of chromosome 11 which encompassws 11p15.5, is associated not only with embryonal rhabdomyosarcomas (Scrable et al., 1990) but, also with a number of other solid tumors (Newsham et al., 1991). This suggests that the location of a tumor suppressor gene(s) for multiple tumor types is in this region. In addition, gene amplifications have been observed in both embryonal and alveolar rhabdomyosarcomas. Utilizing Comparative Genomic Hybridization (CGH) on primary alveolar rhabdomyosarcomas, the most frequent amplicons have been localized to 2p24 and 12q13-15 with both amplifications occurring in 4 out of 10 tumors (Weber-Hall et al., 1996). The 2p24 amplicon had previously been shown to involve the MYCN gene

(Dias et al., 1990), while the genes involved in the 12q13-14 amplicon have not yet been fully defined. Two distinct chromosome 12q13-14 amplification units have been described in other types of sarcomas (Suijkerbuijk et al., 1994; Tarkkanen et al., 1995), as well as in gliomas (Reifenberger et al., 1994). In addition, mapping of these two amplicons implicates MDM2 or CDK4 and SAS as likely targets of the amplification events (Reifenberger et al., 1994).

One obvious phenotype of tumor cells is a lack of terminal differentiation. Recently, we initiated a series of somatic cell genetic experiments designed to identify genetic loci, present in rhabdomyosarcoma cells lines, capable of inhibiting muscle differentiation (Fiddler et al., 1996). We have shown that transfer of a derivative chromosome 14, from the alveolar rhabdomyosarcoma cell line Rh18, into the differentiation competent myoblast cell line C2C12 inhibits both muscle differentiation and the ability of MyoD to trans-activate reporter constructs. The derivative 14 contains a region of amplified DNA originating from chromosome 12q13-14, as well as, a number of genes often amplified in sarcomas. By testing the amplified genes for the ability to inhibit muscle-specific gene expression, forced expression of MDM2 was found to inhibit MyoD function, and consequently prevent muscle differentiation. Thus, amplification and overexpression of MDM2 inhibits MyoD function and results in a dominant inhibition of muscle differentiation (Fiddler et al., 1996).

The oncogenic properties of MDM2 have been postulated to result from direct interaction with a number of cell cycle regulatory proteins. MDM2 interacts directly with p53 (Oliner et al., 1992), and blocks p53 mediated transactivation by inhibiting the activation domain of p53 (Chen et al., 1993; Haines et al., 1994; Momand et al., 1992; Oliner et al., 1993; Wu et al., 1993; Zauberman et al., 1993). The crystal structure of the amino-terminal domain of MDM2 bound to the activation domain of p53 has recently been described (Kussie et al., 1996). The model suggests that MDM2 contains a deep hydrophobic cleft on which the p53 activation domain binds as an amphipathic α helix.

In addition, MDM2 has been shown to interact directly with pRb, resulting in stimulation of E2F/DP1 transcriptional activity and inhibition of pRb growth regulatory function (Xiao et al., 1995). Furthermore, MDM2 interacts with the activation domain of E2F1 resulting in stimulation of E2F1/DP1 transcriptional activity (Martin et al., 1995). Taken together, these results suggest that MDM2 not only relieves the proliferative block mediated by either p53 or pRb, but also promotes proliferation by stimulating the S-phase inducing transcriptional activity of E2F/DP1. In addition, over-expression of E2F1 in C2C12 and 10T1/2 cells resulted in apoptosis (Fiddler, unpublished). If the ability of MDM2 to inhibit muscle-specific gene expression was through the E2F1 pathway, then it appears that effects must occur without an increase in E2F1 levels. In fact, northern analysis indicates that E2F1 levels are essentially the same within all the generated microcell hybrids and rhabdomyosarcoma cells (Fiddler, unpublished).

Results

Amplification of MDM2 is a Common Event

We have previously shown that the rhabdomyosarcoma cell line Rh18 contains an amplification of MDM2 (Fiddler et al., 1996). In addition, MDM2 was also shown to be amplified in a case of primary rhabdomyosarcoma (Meddeb et al., 1996). However, it is currently not known how frequently this amplification occurs in primary tumors. To address this issue, we assayed for MDM2 DNA amplification in primary rhabdomyosarcomas by Southern blot hybridization. Figure 2-1 shows a representative autoradiogram showing amplification of MDM2 in primary rhabdomyosarcoma samples. Overall, we have found that MDM2 is amplified in 10 of the 22 primary rhabdomyosarcomas screened, and therefore represents a common genetic abnormality in this type of tumor. This frequency is consistent with the observed frequency of 12q13-14 amplifications in rhabdomyosarcomas detected using CGH (Weber-Hall et al., 1996).

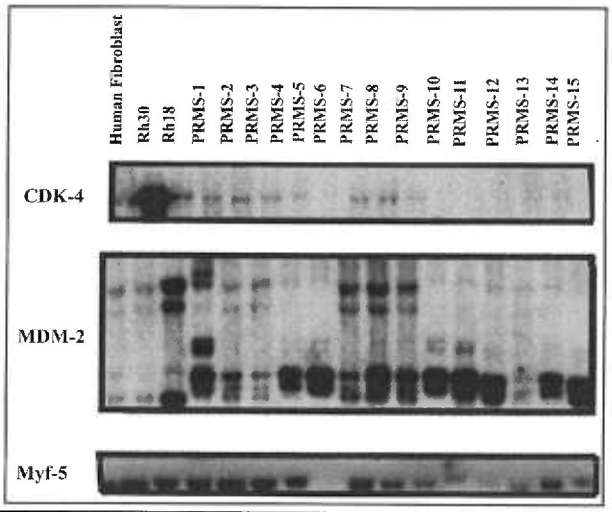

Figure 2-1. MDM2 is amplified in Rh18 and primary rhabdomyosarcomas, but not Rh30. Southern hybridization with full-length MDM2 cDNA, full-length CDK-4 cDNA and full-length myf-5 cDNA, respectively. Ten micrograms of genomic DNA was extracted from primary human rhabdomyosarcomas (PRMS), Rh18, Rh30, and human fibroblast.

However, because CDK4, but not MDM2, is amplified in the rhabdomyosarcoma cell line Rh30 [see below and (Khatib et al., 1993)], and because CDK4 was identified as a likely target of the amplification events in other types of tumors (Reifenberger et al., 1994), we tested for amplification of CDK4 in this set of rhabdomyosarcomas. Figure 2-1 shows a representative Southern blot showing amplification of CDK4 in the rhabdomyosarcoma cell line Rh30 but not in the fourteen primary rhabdomyosarcomas. Although Rh30 was the only rhabdomyosarcoma cell line harboring a CDK4 amplification, Rh18 microcell hybrids carrying the derivative 14 chromosome demonstrated elevated levels of CDK4 transcripts compared to that of normal human myoblasts. Figure 2-3 illustrates Rh30 as having four fold the amount of CDK4 RNA as that found in the other rhabdomyosarcoma cell lines. This indicates that a genomic amplification of CDK4 is not necessary for overexpression of the gene. We did not detect amplification of CDK4 in any of the primary rhabdomyosarcomas screened. Thus, we conclude that amplification of MDM2 is a frequent event in rhabdomyosarcomas and that amplification of CDK4 must be a more rare event.

Amplification of several genes from the region 12q13-14 has been reported in sarcomas (Forus et al., 1993; Nilbert et al., 1994), as well as, in gliomas (Reifenberger et al., 1994). Two distinct chromosome 12q13-14 amplification units can be found in these tumors, implicating MDM2 or CDK4 and SAS as the likely targets of the amplification (Reifenberger et al., 1994). These studies have elicited two main hypotheses: either

different oncogenes are selectively amplified in different tumors, or an as yet unidentified oncogene located in the 500-kb region between MDM2 and CDK4 is the target of these amplification events. We have found that several genes from chromosome 12q13-14, including MDM2, GLI, SAS and CHOP are co-amplified in the rhabdomyosarcoma cell line Rh18 (Figure 2-2). Previous studies identified 12q13-14 DNA amplification, containing amplified CDK4 and GLI, in the rhabdomyosarcoma cell line Rh30 [(Khatib et al., 1993) see Figure 2-1]. Rh18 and Rh30 cells contain distinct 12q13-14 amplicons. Rh18 cells contain amplified MDM2 but not CDK4, and Rh30 cells contain amplified CDK4 but not MDM2 (Figure 2-1). If amplification of an as yet unidentified oncogene is the target of amplification in this region, we would predict that, like the Rh18 amplification ([Dias, 1990 #58) , transfer of the Rh30 12q13-14 amplification into C2C12 myoblasts would inhibit muscle differentiation. Furthermore, because overexpression of the CDK4 partner cyclin-D1 inhibits muscle differentiation (Skapek et al., 1995), amplification of CDK4 may also inhibit muscle differentiation. To test these possibilities, we transferred Rh30 chromosomes, tagged by transfection with a neo^r vector, into C2C12 cells by microcell

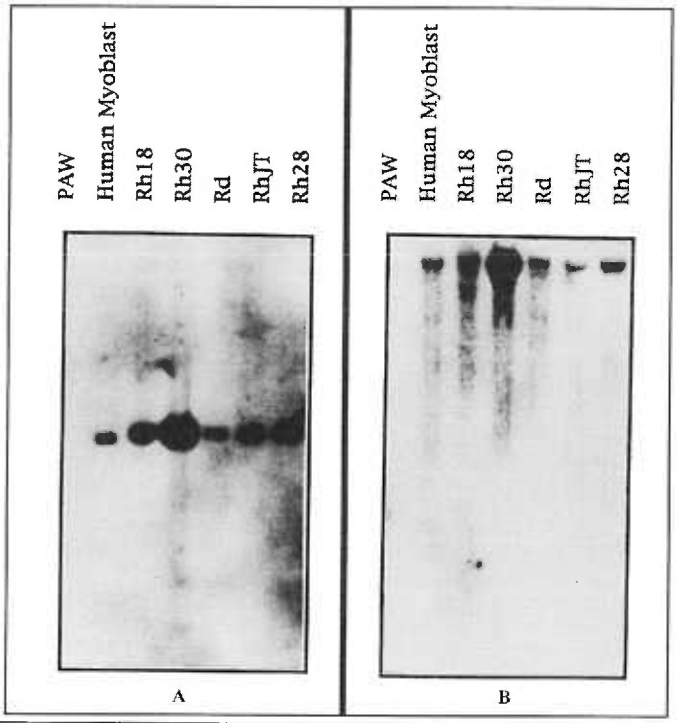

Figure 2-2. Amplification of SAS and Gli oncogenes in rhabdomyosarcoma cell lines. Southern blot hybridizations with SAS PCR product as a probe (A) and Gli PCR product as a probe (B) respectively. Ten micrograms of genomic DNA was extracted from human myoblast, Rh18, Rh30, RD, RhJT, and Rh28. PAW, molecular weight marker.

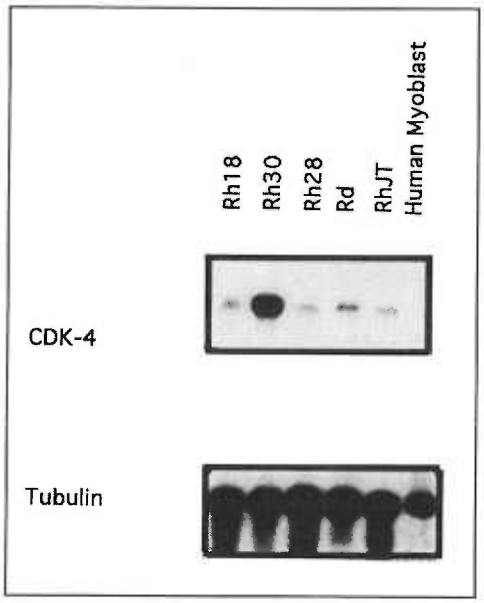

Figure 2-3. CDK-4 is overexpressed in Rh30, not Rh18. Northern blot hybridization to 5.0 μg of cytoplasmic RNA extracted from human myoblast, Rh18, Rh28, Rh30, RhJT, and RD. The probes were full-length CDK-4 cDNA or α-tubulin. CDK-4 transcripts are detected from human myoblast following a longer exposure (not shown).

fusion and screened the resulting primary microcell hybrids for retention of amplified GLI and CDK4 by Southern blot hybridizations. This screen identified three clones that retained this amplification, C2 (RH30)-3, -11 and –12, detected by Southern blot hybridization, using mouse Cdk4 as probe, on DNAs from these three microcell hybrids. Visual inspection of these three hybrids under differentiation inducing conditions revealed extensive cell fusion and myotube formation in all three clones.(Thayer, unpublished) Because all of the primary microcell hybrid clones from a similar fusion, using Rh18 cells as donors, resulted in transfer of 2-3 human chromosomes (Fiddler et al., 1996), we conducted a second round of microcell fusion. Each primary microcell hybrid was utilized as donor in microcell fusions with C2C12 cells, again selecting for transfer of neo^r by growth in media containing G418. The resulting hybrids were assayed for retention of amplified CDK4 as well as for muscle differentiation, as described above. All three primary microcell hybrid clones transmitted the amplified chromosome with the neo^r marker in five out of five secondary clones tested, indicating that the neo^r insertions in all three primary clones were linked to the amplification. Northern blot hybridizations, using MyoD, myogenin, myosin light chain 1/3 (MLC) and tubulin as probes on RNA extracted from parental C2C12 cells and three secondary microcell hybrids was performed(Thayer,unpublished). Consistent with the myotube formation observed in the primary hybrids, all three secondary hybrids express

high levels of MyoD, myogenin and MLC mRNA. This result indicates that, in contrast to the MDM2 amplification found in Rh18 cells, the Rh30 chromosome 12 DNA amplification does not inhibit muscle differentiation when transferred into C2C12 cells. To address this directly, we co-transfected CDK4 and muscle specific reporter MCK-CAT into C2C12 myoblasts (data not shown). Over-expression of CDK4 had no inhibitory effects on transcription from MCK-CAT. In addition, these data indicate that two separate regions of 12q13-14 can be amplified in rhabdomyosarcoma cell lines and that these distinct amplicons result in different phenotypes. In summary, genetic aberrations resulting in the overexpression of CDK4 alone are insufficient in abolishing the muscle phenotype, while amplification and overexpression of MDM2 results in an inhibition of muscle differentiation.

MDM2 Mediated Transcriptional Repression is Independent of p53

Mutation of p53 is the most common genetic event in the development of human neoplasia, occurring in approximately 50% of most types of tumors (Hollstein et al., 1991). It has been proposed that MDM2 functions as a feedback regulator of p53 action by being transcriptionally induced by p53 (Wu et al., 1993) and then inhibiting p53 function (Momand et al., 1992; Oliner et al., 1993; Wu et al., 1993). To begin to analyze the effects of MDM2 amplification on the cell cycle, we first tested whether forced expression of p53 could rescue the MDM2 repression of muscle gene transcription. Constitutive expression of wild-type p53 had no positive effects on muscle-specific gene transcription from the non-muscle hybrids, or C2C12 and 10T1/2 cells co-transfected with MDM2. Because MDM2 is capable of inhibiting p53 dependent transcription (Momand et al., 1992), we wanted to determine whether the p53 induced p21 would have an effect on muscle gene transcription in the presence of elevated levels of MDM2.

Similar to the p53 data, expression of p21 failed to rescue muscle gene transcription in the microcell hybrids (data not shown).

Three observations led us to postulate that inhibition of myogenesis by MDM2 may involve inactivation of p53: 1) amplification of MDM2 results in loss of p53 and MyoD functions (see above); 2) expression of the CDK inhibitor p21 is induced by both p53 and MyoD, but is not induced in the presence of amplified MDM2, and 3) p53 response elements have been found in the control region of at least one muscle-specific gene, the muscle creatine kinase (MCK) gene (Weintraub et al., 1991; Zambetti et al., 1992). Furthermore, a link between MDM2 and p53 during development has recently been demonstrated by generating MDM2 and p53 null mice. Homozygous knockout of MDM2 results in an early embryonic lethal phenotype that is rescued by deletion of p53 (de-Oca-Luna et al., 1996; Jones et al., 1995). This result suggests that MDM2 relieves the cell cycle block mediated by p53 early in development. Therefore, to determine whether p53 is required for inhibition of MyoD activity by MDM2, we assayed the ability of MDM2 to inhibit MyoD transactivation in p53 null fibroblasts. These experiments demonstrated that MyoD is still able to trans-activate its target site (4RCAT) in the absence of p53, and that MDM2 is still capable of inhibiting this activation. In addition, these data are consistent with the observation that forced expression of neither p53 nor p21 in the derivative 14 hybrids is able to restore MyoD activity (not shown). These data are also consistent with previous observations showing that loss of the p53 gene does not lead to loss of MyoD function (Halevy et al., 1995; Parker et al., 1995). Taken together, these results indicate that MyoD activity does not require p53, and that inhibition of MyoD activity by MDM2 also does not require p53.

Transcriptional Inhibition is DNA Site Specific

To determine what portion of MyoD is the target of MDM2 inhibition, we assayed GAL-MyoD fusion constructs (Figure 2-4) for sensitivity to MDM2 inhibition. Figure 2-4A shows that MDM2 does not inhibit the activation domain of MyoD fused to the GAL4 DNA binding domain (GAL-N62) when assayed on the GAL4 dependent reporter GALCAT. Furthermore, MDM2 does not inhibit transactivation of full length MyoD fused to the DNA binding domain of GAL4 (GALMyoD) when assayed on the GALCAT reporter. However, MDM2 is still capable of inhibiting GALMyoD mediated transactivation when assayed on the MyoD dependent reporter constructs MCKCAT and 4RCAT (Figure 2-4B). This result indicates that inhibition by MDM2 involves the MyoD DNA binding domain and the MyoD DNA binding site. Furthermore, MDM2 is able to inhibit the transcriptional activity of the MyoD-VP16 fusion protein, specific to MyoD not the VP16 domain (Figure 2-4A, 2-4C). These results also indicate that inhibition of MyoD activity by MDM2 does not involve loss of MyoD protein, since GALMyoD is fully functional on the GAL4 DNA binding sites in the presence of MDM2. Furthermore, utilizing gel mobility shift assays, combined with antibody "supershift" experiments, we find equivalent amounts of MyoD DNA binding activity in the presence of amplified MDM2 (not shown). Because MDM2 binds and inhibits the activation domain of p53 (Oliner et al., 1993), and MDM2 inhibits GALMyoD activity only on MyoD dependent reporters, we conclude that the mechanism of inhibition of MyoD activity by MDM2 must be different than for inhibition of p53 activity. Recent studies have shown that many of the cellular effects, which are mediated by MDM2, occur through distinct protein-protein interactions(Haines et al., 1994; Martin et al., 1995; Xiao et al., 1995). In order to determine whether MDM2 and MyoD protein interactions exists, immunoprecipitation experiment where carried out using *in vitro* translated MDM2 and MyoD. No protein-protein interaction was detected using either polyclonal

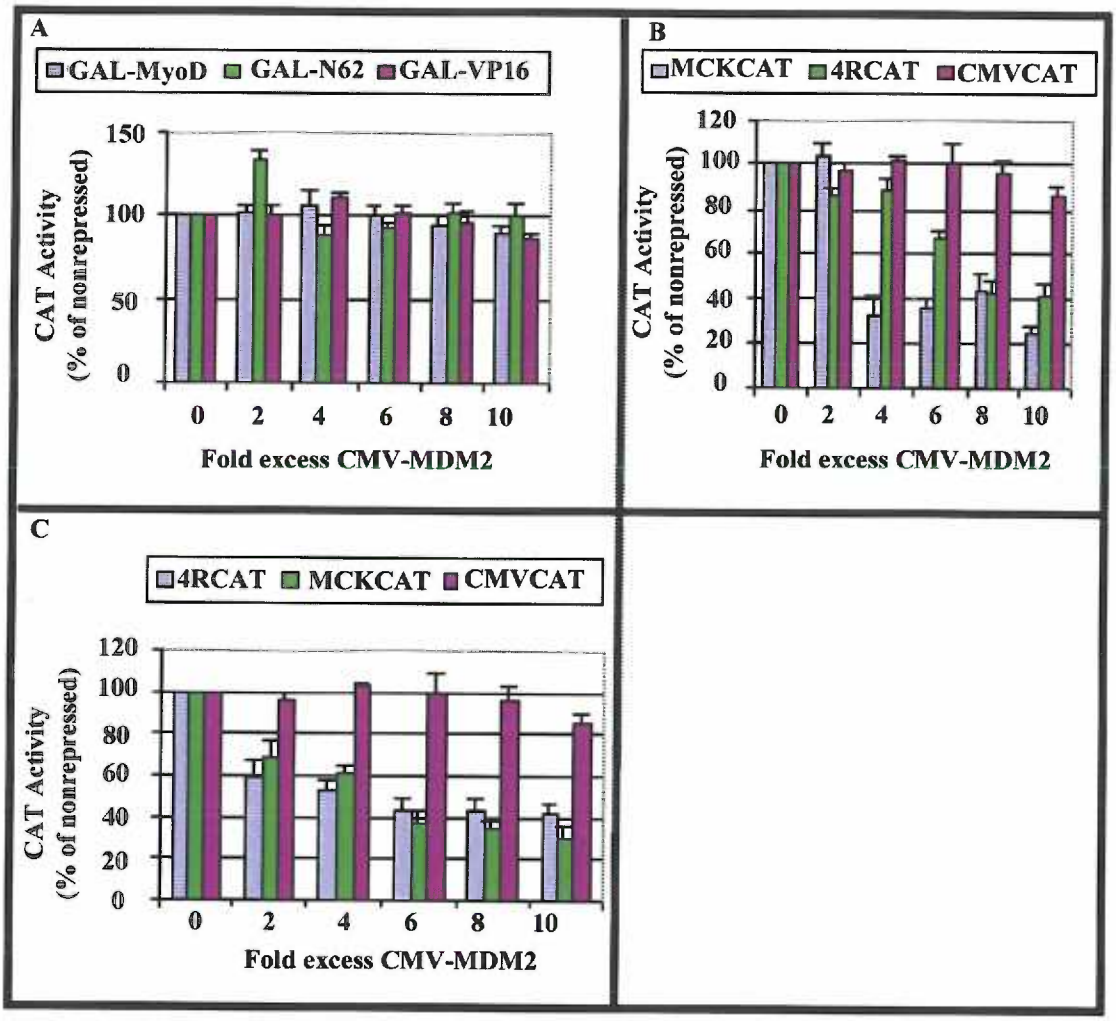

Figure 2-4. MDM2 repression of MyoD-dependent transcription is DNA site dependent. (A) 10T1/2 cells were transfected with 0.5 μg GAL-MyoD, GAL-N62, or GAL-VP16 expression vectors, 0.5 μg GAL-CAT reporter construct, and increasing amounts of MDM2. (B) 10T1/2 cells were transfected with 0.5 μg GAL-MyoD expression vector, 0.5 μg of the indicated reporter constructs, and increasing amounts of MDM2. (C) 10T1/2 cells were transfected with 0.5 μg MyoD-VP16 expression vector, 0.5 μg of the indicated reporter constructs, and increasing amounts of MDM2. The values for all the above represent the averages from three separate experiments, with each experiment containing duplicate dishes. Duplicate dished showed less than 10% variability. All transfections were kept at a total of 6.0 μg DNA by substituting an empty expression vector for the MDM2 expression construct.

sera specific to MyoD, nor Polyclonal or monoclonal antibody directed at MDM2. In the event that a potential interaction required the MyoD dimerization partners translated E12 protein was included in the immunoprecipitation reactions. Neither co-translated nor single translated products demonstrated protein-protein interaction between MyoD and

MDM2. In the event that post-translational modification may be necessary for protein interactions immuno-precipitations were done on whole cell and nuclear extracts from both C2C12 and 10T1/2 cells which where co-transfected with MyoD, MDM2 and E12. Similar to the *in vitro* data, no protein interaction was detected between MyoD and MDM2. In addition, yeast two-hybrid assays using MyoD and MDM2 fusion proteins failed to indicate the protein interaction exists. This suggests that the MDM2 inhibition of MyoD function occurs by an indirect mechanism, rather then direct protein-protein interaction.

Rb Rescues Muscle Gene Transcription

It is currently believed that the members of the Rb family modulate cell cycle progression by negatively regulating the transcriptional activity governed by E2F/DP1 heterodimers which promote DNA synthesis and cell growth (Cress et al., 1993; DeGregori et al., 1995; Helin et al., 1992; Hiebert et al., 1992; Lam and La-Thangue, 1994; Picksley and Lane, 1994). Current evidence demonstrates the Rb and MyoD interact *in vitro* (Gu et al., 1993; Novitch et al., 1996), although it is unclear whether this direct interaction is required for either protein to function. Rb function is apparently required to inhibit DNA synthesis in differentiated cells (Endo and Goto, 1992). Given that MDM2 was recently shown to interact with Rb (Xiao et al., 1995), we were interested in whether expression of Rb could rescue the MDM2 inhibition of muscle gene transcription. Expression of Rb in C2C12 cells failed to alleviate the MDM2 repression on MCK-CAT beyond sixty percent of the activity of non-repressed C2C12 cells (Figure 2-5). Whereas, transfection of Rb into the non-muscle forming hybrids resulted in a slight increase in expression from both 4RtkCAT and MCK-CAT reporter constructs culminating at levels which where approximately ten to fifteen percent of that found in C2C12 cells. The relatively minor effect which Rb had on MDM2 repression of muscle

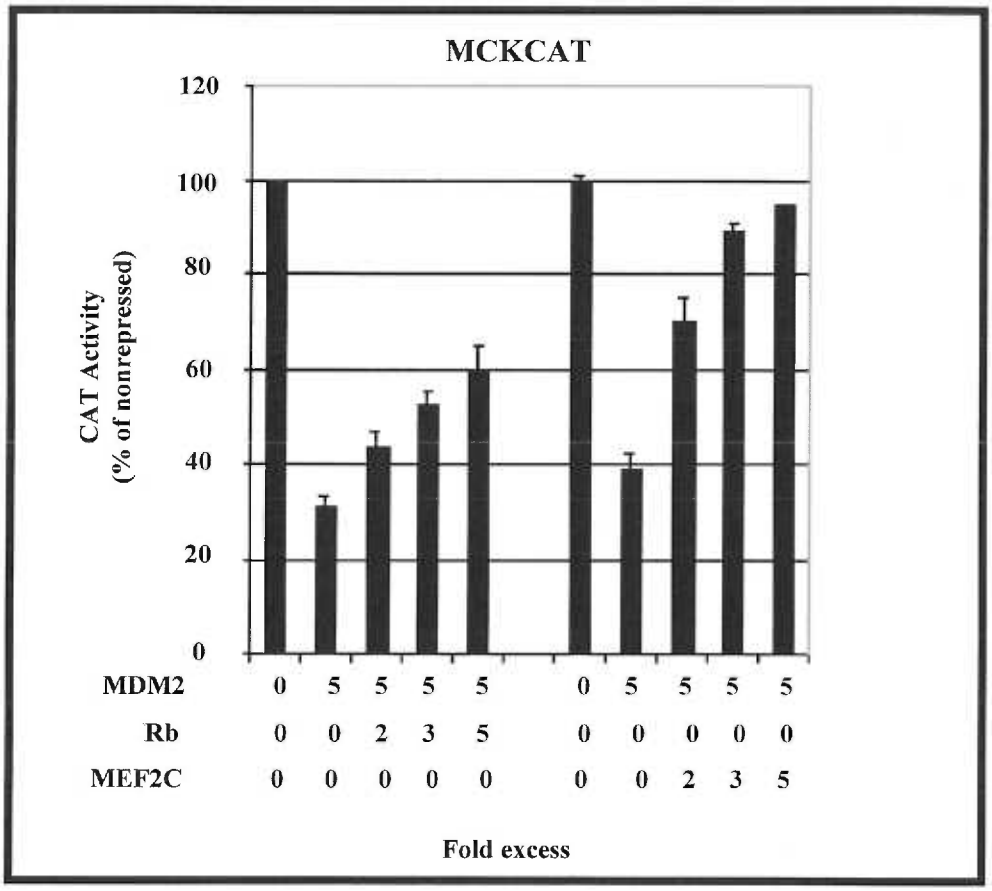

Figure 2-5. Rescue of MCKCAT activity by MEF2C and Rb. C2C12 cells were transfected with 0.5 μg of the muscle specific reporter construct MCKCAT, and the indicated fold excesses of MDM2, MEF2C, and Rb. Increasing amounts of the expression vectors indicated as fold excess with respect to the reporter construct. The values represent the averages of at least two separate experiments, with each experiment containing duplicate dishes. All transfections were kept are a total of 6.0 μg DNA by substituting an empty expression vector of the gene expression constructs.

gene transcription is most likely do to Rb binding and sequestering the MDM2 protein from interacting with muscle regulator proteins (see below). The restoration of muscle-specific gene transcription in C2C12 by Rb may be an indication of a crucial role played by Rb in maintaining myoblasts in a differentiated state. In addition, expression of p107 and p130, two members of the RB family failed to rescue the repression on muscle gene transcription imposed by excess MDM2. Both p107 and p130 have been demonstrated to interact with E2F (Cress et al., 1993; Helin et al., 1992; Hiebert et al., 1992; Picksley and

Gene Expression in Rhabdomyosarcomas and Microcell Hybrids											
	p53	p21	p57	p16	CDK4	E2F-1	Cyclin A	Cyclin D1	pRb	p107	MDM2
Rh18	+	+	+	+	+	+	+	+	+	+	+++++
Rh30	+	+	+	+	++++	+	+	+	+	+	+
Rh28	+	+	+	+	+	+	+	+	+	+	+
RhJT	+	+	+	+	+	+	+	+	+	+	+
RD	+	+	+	+	+	+	+	+	+	+	+
H.Myoblast	+	+	+	+	+	+	+	+	+	+	+
H.Fibroblast	+	+	+	+	+	+	+	+	+	+	+
C2C12	+	+	+	+	+	+	+	+	+	+	+
C2(Rh1811)-4	+	+	+	+	+	+	+	+	+	+	+
C2(Rh1811)-6	+	+	+	+	++	+	+	+	+	+	+++++
C2hiR1811-E	+	+	+	+	+	+	+	+	+	+	+
C2hiR1811-O	+	+	+	+	++	+	+	+	+	+	+++++

Table 2-1. Expression of cell cycle genes in rhabdomyosarcoma cell lines and microcell hybrid cell lines. Data depicted in the table is the result of Northern blot hybridization experiments using the indicated genes is DNA probes. The (+) symbols indicate the relative amount of transcripts detected with a single (+) being equivalent to a single level of expression. Multiple (+) marks indicate fold over expression (i.e. (+++) indicated three-fold over expression). All Northern hybridizations were carried out as described in Methods.

Lane, 1994; Schneider et al., 1994), and are postulated to restrain E2F mediated transcriptional activity in a similar manner to which Rb inactivates E2F proteins. Furthermore, northern analysis revealed that C2C12, non-muscle forming hybrids and muscle forming hybrid expressed equivalent levels of p107, p130 and pRb (Fiddler, unpublished) (Table 2-1). This is suggestive that the negative regulatory effects orchestrated by MDM2 on the muscle program are independent actions governed by the Rb family of tumor suppressor genes.

MEF2-C Expression Rescues Muscle-Specific Gene Transcription

Recently, p300 was shown to be required for MyoD dependent cell cycle arrest and muscle-specific gene transcription (Puri Pieri, 1997). The p300 protein belongs to a family of transcriptional co-activators that are able to modulate transcription. Co-activators function as adapter proteins for complex transcriptional regulatory elements by enhancing the communication between specific transcription factors and components of

the transcriptional machinery. Both p300 and the related protein CBP have been demonstrated to co-activate myogenic bHLH proteins by a mechanism that involves a direct association of p300 with the amino-terminal domain of MyoD (Eckner et al., 1996). In addition, p300 and CBP (CREB binding protein) serve as transcriptional co-activators of MEF-2C by direct interaction with the MADS domain of the MEF-2C molecule (Sartorelli, 1997; Yuan, 1996). Given that p300 effectively synergizes myogenic bHLH function, a potential mechanism by which MDM2 halts muscle-specific gene expression would be through inactivation of p300 function. To test this transient transfection assays were carried out in the non-muscle forming hybrids. Both p300 and CBP failed to rescue expression from MCK-CAT and 4Rtk-CAT in microcell hybrid C2 (Rh1811)-6. Likewise, co-transfection of p300 and MDM2 in 10T1/2 and C2C12 cells lacked the ability to inhibit the MDM2 repression of muscle gene transcription. Although p300 is a common co-activator of myogenic bHLH proteins and MEF-2C, it appears that MDM2 extinguishes the muscle program through another pathway then that which is governed by p300 activity.

Muscle-specific transactivation by MyoD has been shown to require a second factor (a "recognition factor") that recognizes the MyoD basic region bound to DNA (Davis et al., 1990; Weintraub et al., 1991). Recently, members of the MEF2 family of muscle-specific transcription factors have been shown to act synergistically with MyoD to induce myogenesis (Kaushal et al., 1994; Molkentin et al., 1995). Both MEF2A and MEF2C bind to the b-HLH domain of MyoD and facilitate transcriptional activation through a MyoD DNA binding site. Furthermore, MEF2C interacts with MyoD mutants that retain myogenic activity and not with MyoD mutants that have lost the ability to induce myogenesis (Molkentin et al., 1995), thus MEF2C displays all of the properties of the "recognition factor". Because MDM2 can inhibit the activity of the GALMyoD construct in a DNA site-specific manner (i.e. only on the MyoD binding site and not on the GAL4 binding site; Figure 2-4), we postulated that MDM2 may function by

inactivating MEF2C. Therefore, we next determined whether forced expression of MEF2C could restore MyoD activity in cells expressing MDM2. Figure 2-5 shows that co-transfecting increasing amounts of the MEF2C expression vector restores MyoD activity in the presence of high levels of MDM2 in C2C12 cells. Transfection of MEF2C into non-muscle microcell hybrids resulted in an increase in expression from MCK-CAT to levels comparable to twenty-five percent of the activity in C2C12. These results suggest that MDM2 is inhibiting MyoD activity indirectly, and that MEF2C is a likely target of MDM2 action.

Discussion

Rhabdomyosarcomas arise *de novo* from skeletal muscle, and in some instances demonstrate morphological characteristics of differentiated muscle tissue, but do not undergo overt differentiation. We previously utilized a genetic screen to identify loci, present in the rhabdomyosarcoma cell line Rh18, capable of inhibiting muscle differentiation (Fiddler et al., 1996). This screen identified a DNA amplification, originating from chromosome 12q13-14 capable of inhibiting MyoD activity and muscle differentiation. Testing the amplified genes for the ability to inhibit muscle-specific gene expression indicates that forced expression of MDM2 inhibits MyoD function, and consequently inhibits muscle differentiation. Testing primary rhabdomyosarcomas for DNA amplifications indicates that amplification of MDM2 is a frequent event, occurring in 45% of the cases. Furthermore, we have shown in this study that two distinct 12q13-14 amplicons can be detected in rhabdomyosarcoma cell lines and that these amplicons result in different phenotypes. Amplicons containing MDM2 result in loss of p53 activity as well as loss of terminal differentiation. In contrast, the amplicon present in Rh30 cells, which contains amplified CDK4 and GLI, does not result in loss of p53 activity nor loss of terminal differentiation. The phenotypic consequences of the Rh30 amplification

remain to be elucidated; however, analysis of the microcell hybrids generated in this study should allow for any phenotypic changes associated with amplified CDK4 to be defined. Furthermore, amplification of CDK4 was not detected in any of the primary rhabdomyosarcomas tested in this study. Thus, amplification of CDK4 must be a rare event in this type of tumor.

Forced expression of p53 failed to rescue the non-muscle phenotype displayed by the microcell hybrids. In addition, expression the p53 effector had no noticeable effect on the level of muscle gene transcription in the presence of high MDM2 levels. These results are consistent with the observation that p53 is not required for the inhibition of MyoD activity by MDM2. Our data are also consistent with previous observations showing that loss of the p53 gene does not lead to loss of MyoD function (Halevy et al., 1995; Parker et al., 1995). Taken together, these results indicate that MyoD activity does not require p53, and that inhibition of MyoD activity by MDM2 also does not require p53.

Differentiating muscle cells fuse to form multinucleated myotubes and permanently withdraw from the cell cycle. This process is controlled by regulatory interactions involving MyoD family members and various cell cycle related proteins [reviewed in (Lassar et al., 1994; Olson, 1992). Myf-5 and MyoD are present in proliferating myoblasts and initiate a cascade of events resulting in terminal differentiation when myoblasts are deprived of mitogens. Several hypotheses have been proposed to explain how the MyoD family of proteins are kept in check during proliferation: 1) inhibition of the MyoD family members by interaction with the Id family of negative HLH factors (Benezra et al., 1990), 2) inhibitory phosphorylation of the MyoD family members (Hardy et al., 1993; Li et al., 1992), and more recently 3) inhibition by cyclin-D dependent kinases (Rao et al., 1994; Skapek et al., 1995). In addition, MyoD transactivation of muscle-specific genes to high levels requires pRB (Gu et al., 1993; Novitch et al., 1996) or high levels of the pRB related protein p107 (Schneider et al., 1994). Furthermore, during muscle differentiation, Rb expression

increases (Coppola et al., 1990; Endo and Goto, 1992; Martelli et al., 1994; Schneider et al., 1994) and assumes a hypophosphorylated, activated state (Gu et al., 1993; Thorburn et al., 1993). Since MDM2 interacts with RB and inhibits the growth regulatory functions of RB (Xiao et al., 1995), and MDM2 is expressed in proliferating myoblasts (Fiddler et al., 1996), it seems reasonable to speculate that RB may in turn regulate the growth stimulatory affects of MDM2. This notion is supported by the observation that forced expression of RB in our hybrid cells containing amplified MDM2 results in enhanced muscle differentiation (Fiddler, unpublished). Thus, induction and activation of RB during muscle differentiation may result in inhibition of MDM2 activity thereby allowing MEF2C to function and synergize with MyoD, resulting in terminal muscle differentiation. Figure 2-6 illustrates potential mechanisms by which MDM2 can inhibit muscle cell differentiation.

The ability of cells to control precisely the order and timing of cell cycle events is important for the maintenance of genomic stability and the prevention of mutations that can disrupt normal growth control [review by (Fiddler et al., 1996; Hartwell, 1992; Hartwell and Kastan, 1994)]. For example, in response to DNA damage, cells arrest cell cycle progression and induce the expression of gene products that facilitate DNA repair. In response to DNA damage, p53 levels increase resulting in transcriptional activation of p53 target genes [recently reviewed in (Ko and Prives, 1996)]. Induction of the cyclin

The ability of cells to control precisely the order and timing of cell cycle events is important for the maintenance of genomic stability and the prevention of mutations that can disrupt normal growth control [review by (Fiddler et al., 1996; Hartwell, 1992; Hartwell and Kastan, 1994)]. For example, in response to DNA damage, cells arrest cell cycle progression and induce the expression of gene products that facilitate DNA repair. In response to DNA damage, p53 levels increase resulting in transcriptional activation of p53 target genes [recently reviewed in (Ko and Prives, 1996)]. Induction of the cyclin dependent kinase inhibitor $p21^{CIP1/WAF1}$ by p53 following DNA damage results in G1

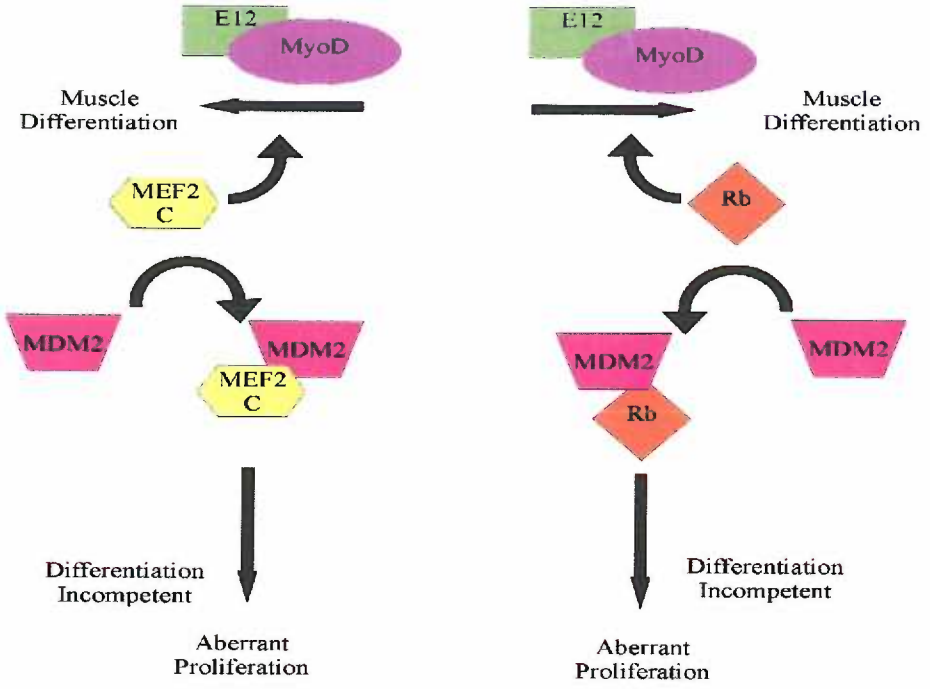

Figure 2-6. Model for MDM2 Inhibition of Muscle Differentiation
Overexpression of MDM2 results in and increase in cellular protein levels of MDM2. Excess MDM2 can potentially interact with factors required for muscle differentiation. MDM2 binding to MEF2-C can result in inhibition of the synergesim between the MyoD family and MEF2-C resulting in myocytes that are differentiation incompetent. In addition, MDM2 may bind Rb and inactivate the Rb growth suppressing function. Furthermore, MDM2 interaction with Rb may abolish Rb's interaction with MyoD and thus inhibit muscle differentiation.

arrest (Deng et al., 1995). Furthermore, $p21^{CIP1/WAF1}$ is induced by MyoD during muscle differentiation (Halevy et al., 1995), and forced expression of $p21^{CIP1/WAF1}$ in serum stimulated myoblasts causes premature differentiation (Skapek et al., 1995). Thus, a prediction based on these observations would be that DNA damage would induce serum stimulated myoblasts to differentiate by increasing $p21^{CIP1/WAF1}$ levels. However, even though $p21^{CIP1/WAF1}$ is induced in proliferating myoblasts following DNA damage, these cells do not undergo premature differentiation following γ irradiation (Thayer, unpublished). An attractive solution to this paradox is the observation that DNA damage induces MDM2 in a p53 dependent manner (Barak et al., 1993; Wu et al., 1993). Thus, DNA damage induces $p21^{CIP1/WAF1}$ expression, but the cells fail to differentiate because MDM2 is also induced, consequently inhibiting MEF2C function. A role for MDM2 during normal muscle differentiation, as well as following DNA damage is currently under investigation.

Conclusions

One obvious phenotype of tumor cells is a lack of terminal differentiation. Previously, we classified rhabdomyosarcoma cell lines as having either a recessive or a dominant non-differentiating phenotype (Tapscott et al., 1993) . To study the genetic basis of the dominant non-differentiating phenotype of Rh18 cells, we utilized microcell fusion to transfer chromosomes from Rh18 cells into the mouse myoblast cell line C2C12. Rh18 chromosomes were tagged with a selectable marker and transferred into C2C12 cells. Two different non-muscle phenotypes were obtained: one characterized by a complete lack of expression of MyoD and myogenin, and another, characterized by continued expression of MyoD and myogenin. Karyotypic analysis indicates that a derivative chromosome 14 is responsible for the MyoD and myogenin positive non-differentiating phenotype. The derivative chromosome 14 contains an amplification comprised of chromosome 12 DNA sequences. MDM2 is amplified and overexpressed in Rh18 and in the non-differentiating microcell hybrids that retain the derivative 14. Forced expression of MDM2 in C2C12 cells, and in 10T1/2 cells transfected with a MyoD expression vector results in repression of MyoD dependent transcription. In addition, expression of anti-sense MDM2, in C2C12 hybrids containing the derivative 14, results in restoration of MyoD dependent transcriptional activity and therefore restoration of muscle-specific gene expression. We conclude that amplification and overexpression of MDM2 in rhabdomyosarcomas leads to inhibition of MyoD dependent transcription and results in a dominant non-differentiating phenotype.

In contrast to normal diploid cells which lack detectable gene amplifications, gene amplifications are easily detected in tumors and transformed cell lines (Tlsty, 1990) . Gene amplifications are often responsible for drug resistance in cultured mammalian cells, while amplification of cellular oncogenes are often observed in tumors. In both

cases, rare clones are selected based on overexpression of genes as a result of increased copy number and is thought to confer a selective growth advantage (Stark et al., 1989) . Detailed mapping studies of amplified DNA indicates that regions far larger than the selected gene are always amplified. The size of amplification units can vary from a few hundred kilobases to as many as 10 megabases (Stark et al., 1989). In addition, other chromosomal abnormalities such as inversions, translocations, and deletions are often observed in conjunction with amplification. Given these observations, perhaps it is not surprising that the DNA amplification described here involves multiple genes encompassing a large region of genomic DNA, as well as two different chromosomes, 12 and 14.

Amplification of cellular proto-oncogenes has been described in a number of different tumor types, including rhabdomyosarcomas (Garson et al., 1986; Garvin et al., 1986; Mitani et al., 1986; Roberts et al., 1989). High frequencies of MDM2 gene amplification has been observed in a variety of tumors, including bone and soft tissue sarcomas (Oliner et al., 1992) and gliomas (Reifenberger et al., 1994) . We have shown here that a number of genes from chromosome 12, including MDM2, GLI, and SAS are co-amplified in the rhabdomyosarcoma cell line Rh18. While it is not presently known how often MDM2 becomes amplified in primary rhabdomyosarcomas, we have found MDM2 DNA amplification in primary rhabdomyosarcomas at a frequency of approximately forty-five percent. Regardless, MDM2 amplification is a common event in soft tissue sarcomas (Khatib et al., 1993; Olson, 1992) and therefore represents a frequent genetic alteration in the generation of tumors. Interestingly, a similar DNA amplification is present in the recessive rhabdomyosarcoma cell line Rh30. In contrast to Rh18

however, Rh30 cells contain amplified GLI and CDK4 but not MDM2 (Khatib et al., 1993). In addition, C2C12 microcell hybrids that retain the Rh30 GLI, SAS, and CDK4 amplified region continue to differentiate into muscle (Thayer, unpublished). This result is consistent with our observation that MDM2 amplification results in a dominant non-differentiating phenotype expressed in Rh18 cells, while Rh30 cells display a recessive phenotype and lack amplification of MDM2. Thus, amplification of GLI, SAS, and CDK4 in Rh30 cells does not result in dominant inhibition of MyoD function. However, the functional consequences of GLI and CDK4 amplification on other tumor phenotypes, such as genomic instability or altered growth rate can be assayed using the system described here. Furthermore, amplification of CDK4 was not detected in any of the screened primary rhabdomyosarcoma in this study. Thus, amplification of CDK4 must be a rare event in this tumor type.

In addition to promoting tumor formation, anchorage independent growth, and cellular immortalization, expression of transforming oncogenes inhibits cellular differentiation in several different cell lineages. In muscle cells, expression of oncogenic tyrosine kinases (v-src and v-fps), growth factor receptors (v-erbB), nuclear oncogenes (v-myc, c-myc, v-erbA, and E1A), and the activated form of signal transducing G proteins (H-ras and N-ras) can inhibit terminal differentiation to varying extents (Falcone et al., 1985; Fiszman and Fuchs, 1975; Gossett et al., 1988; Holtzer et al., 1975; Olson et al., 1987; Payne et al., 1987; Schneider et al., 1987; Webster et al., 1988). It was previously demonstrated that ras and fos prevent myogenesis by inhibiting expression of MyoD (Lassar et al., 1989). Identifying the specific pathway by which each of these

oncogenes and growth factors inhibits myogenesis may provide clues as to how MyoD integrates information relayed *via* various aspects of cellular function.

The oncogenic properties of MDM2 have been postulated to result from direct interaction with a number of cell cycle regulatory proteins. MDM2 interacts directly with p53 (Oliner et al., 1992), and blocks p53 mediated transactivation by inhibiting the activation domain of p53 (Chen et al., 1993; Haines et al., 1994; Momand et al., 1992; Oliner et al., 1993; Zauberman et al., 1993). Forced expression of p53 failed to rescue the non-muscle phenotype displayed by the microcell hybrids. In addition, expression the p53 effector p21 had no noticeable effect on the level of muscle gene transcription in the presence of high MDM2 levels. These results are consistent with the observation that p53 is not required for the inhibition of MyoD activity by MDM2. Our data are also consistent with previous observations showing that loss of the p53 gene does not lead to loss of MyoD function (Halevy et al., 1995; Parker et al., 1995). Taken together, these results indicate that MyoD activity does not require p53, and that inhibition of MyoD activity by MDM2 also does not require p53.

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of negative HLH factors (Benezra et al., 1990), 2) inhibitory phosphorylation of the MyoD family members (Hardy et al., 1993; Li et al., 1992), and more recently 3) inhibition by cyclin-D dependent kinases (Rao et al., 1994; Skapek et al., 1995). In addition, MyoD transactivation of muscle-specific genes to high levels requires pRb (Gu et al., 1993; Novitch et al., 1996) or high levels of the pRb related protein p107 (Schneider et al., 1994). Furthermore, during muscle differentiation, Rb expression increases (Coppola et al., 1990; Endo and Goto, 1992; Martelli et al., 1994; Schneider et al., 1994) and assumes a hypophosphorylated, activated state (Gu et al., 1993; Thorburn et al., 1993). Since MDM2 interacts with Rb and inhibits the growth regulatory functions of Rb (Xiao et al., 1995), and MDM2 is expressed in proliferating myoblasts (Fiddler et al., 1996), it seemed reasonable to speculate that Rb may in turn regulate the growth stimulatory affects of MDM2. This notion is supported by the observation that forced expression of Rb in our hybrid cells containing amplified MDM2 results in enhanced muscle differentiation (Fiddler, unpublished). Thus, induction and activation of RB during muscle differentiation may result in inhibition of MDM2 activity thereby allowing MEF2C to function and synergize with MyoD, resulting in terminal muscle differentiation. In addition, MDM2 has been shown to interact directly with pRb, resulting in stimulation of E2F/DP1 transcriptional activity and inhibition of pRb growth regulatory function (Xiao et al., 1995). Furthermore, MDM2 interacts with the activation domain of E2F1 resulting in stimulation of E2F1/DP1 transcriptional activity (Martin et al., 1995). Taken together, these results suggest that MDM2 not only relieves the proliferative block mediated by either p53 or pRB, but also promotes proliferation by stimulating the S-phase inducing transcriptional activity of E2F/DP1.

The process of muscle differentiation requires the orchestrated expression of multiple genes, MRFs, MEF-2C, Rb, and p21. Differentiated muscle cells fuse to form multinucleated myotubes and permanently withdraw from the cell cycle. This process is controlled by regulatory interactions involving MyoD family members and various cell cycle proteins (Lassar et al., 1994; Olson, 1992) . MEF-2C functions synergistically with the MyoD family and is required for high-level muscle-specific gene expression. Forced expression of MEF-2C in C2C12 cells expressing high levels of MDM2 effectively rescued muscle–specific gene transcription. Furthermore, MDM2 was demonstrated to associate *in vitro* with MEF-2C (Liu, unpublished) but, not with MyoD (Fiddler, unpublished ; Liu, unpublished). Taken together, these results suggest that MDM2 can inhibit muscle gene transcription by inhibition of MEF-2C function.

The ability of cells to control precisely the order and timing of cell cycle events is important for the maintenance of genomic stability and the prevention of mutations that can disrupt normal growth control [review by (Fiddler et al., 1996; Hartwell, 1992; Hartwell and Kastan, 1994)]. For example, in response to DNA damage, cells arrest cell cycle progression and induce the expression of gene products that facilitate DNA repair. In response to DNA damage, p53 levels increase resulting in transcriptional activation of p53 target genes [recently reviewed in (Ko and Prives, 1996)]. Induction of the cyclin dependent kinase inhibitor $p21^{CIP1/WAF1}$ by p53 following DNA damage results in G1 arrest (Deng et al., 1995). Furthermore, $p21^{CIP1/WAF1}$ is induced by MyoD during muscle differentiation (Halevy et al., 1995), and forced expression of $p21^{CIP1/WAF1}$ in serum stimulated myoblasts causes premature differentiation (Skapek et al., 1995). Thus,

a prediction of these observations would be that DNA damage would induce serum stimulated myoblasts to differentiate by increasing $p21^{CIP1/WAF1}$ levels. However, even though $p21^{CIP1/WAF1}$ is induced in proliferating myoblasts following DNA damage, these cells do not undergo premature differentiation following γ irradiation (Thayer, unpublished). An attractive solution to this paradox is the observation that DNA damage induces MDM2 in a p53 dependent manner (Barak et al., 1993; Wu et al., 1993). Thus, DNA damage induces $p21^{CIP1/WAF1}$ expression, but the cells fail to differentiate because MDM2 is also induced, consequently inhibiting MEF2C function.

Summary

Two families of transcription factors, the MyoD group of bHLH muscle regulatory factors and the MEF2 group of MADS-box regulators govern skeletal muscle differentiation (Olson et al., 1995). Members of this network have been shown to interact with each other physically and genetically. The interaction between these proteins results in synergistic activation of the muscle program resulting in positive transcriptional regulation of downstream muscle-specific genes (Molkentin et al., 1995). Once activated the myogenic program has the ability to advocate itself by means of self-regulating expression within members of the MyoD family (Olson, 1992; Sassoon, 1993). The ability to auto activate and cross activate members within the family of transcription factors allows for the firm establishment of the muscle program. The transition to skeletal muscle differentiation is marked by permanent cell cycle arrest, expression of differentiation-specific genes, and fusion between myocytes into mature multinucleated myotubes. Members of the muscle network that are expressed during myoblast proliferation are held in check by diverse mechanisms allowing for population expansion and migration. Alternatively, inappropriate inactivation of the myogenic program results in uncontrolled cellular proliferation and neoplasia.

The MDM2 oncogene has been shown to be amplified in fifteen percent of astrocytomas and glioblastomas (Reifenberger et al., 1994; Reifenberger G, 1996). Amplification of MDM2 has been demonstrated in up to one third of sarcomas (Oliner et al., 1992). Likewise, we have shown MDM2 to be amplified in 45 % of primary rhabdomyosarcomas. This indicates that amplification of MDM2 is a common occurrence

in soft tissue tumors and may be a mechanism by which neoplasia occurs. Further support for the notion of MDM2 involvement in cancer is supported by a recent study describing MDM2 overexpression in 73% of human breast carcinoma (Slamon et al., 1987). Over expression of MDM2 has been well established in leukemias and lymphomas (Chilosi et al., 1994; Haidar MA et al., 1997; Huang et al., 1994), although genetic amplification was not detected, this suggested that some other mechanism, in addition to gene amplification leads to aberrant expression of MDM2. Regardless of the mechanism by which MDM2 mRNA levels increase, a direct correlation has been shown to exist between MDM2 transcript levels and the amount of MDM2 protein within the cell (Bueso-Ramos CE and Glassman A, 1996). We have demonstrated that amplification and overexpression of MDM2 in one rhabdomyosarcoma cell results in the failure to differentiate into mature skeletal muscle.

On initial evaluation, the rhabdomyosarcoma cell line Rh18 was shown to express both myogenin and myf-5 yet, failed to form mature muscle (Tapscott et al., 1993). This appeared to be paradoxical since expression of any member of the MyoD family of proteins in a variety of cell types has the ability to activate the muscle program and orchestrate muscle differentiation. The Rh18 non-muscle phenotype was further demonstrated to be dominant in nature indicating that some factor was present within the Rh18 cells which has the ability to extinguish the muscle program. We chose to use microcell mediated chromosome transfer as a means to isolate the locus responsible for the non-muscle phenotype displayed by the Rh18 cell line. Through three rounds of microcell transfer experiments, using C2C12 myoblasts as recipients, we successfully identified a single chromosome that segregated with the non-muscle phenotype. Both *in*

situ hybridization and southern analysis identified the muscle inhibitor chromosome to be a derivative 14 containing a centrally located HSR region originating from chromosome 12. PCR and Southern evaluation revealed that all chromosome 14 sequences to be single copy, while portions of the HSR region contains amplified sections (Fiddler et al., 1996).

Aberrant DNA amplification has been obsevered in mammalian cells (Stark et al., 1989; Suijkerbuijk et al., 1994). The genes exhibiting amplification typically fall into two categories: drug resistance genes and cellular oncogenes. Physical characterization of the amplicons often revealed rearrangements in addition to amplification of specific regions (Buckler et al., 1991; Forus et al., 1993; Reifenberger G, 1996; Speicher et al., 1995; Weber-Hall et al., 1996). In the case of genes associated with drug resistance, gene duplication followed by enhanced gene expression is equated to a selective growth advantage for those cells that posses the gene amplification. Likewise, an expansion of oncogene number within a cell can function as a positive mode of selection for that clone and all progeny, which also carry the amplified oncogene. Although the mechanism through which gene amplification occur is still unknown, one factor is clear: amplification involves a region of DNA, and not a single gene. In this manner, the simple increase in gene copy number will result in enhanced gene expression. Alternatively, the replication of a gene and its flanking sequences creates repeated genetic units that are devoid of natural sequences that negatively control their transcription. The removal or disassociation from regulatory control sequences most often culminates with an increase in gene expression from those sequences contained within the amplicon. It is simple to imagine how over-expression of a gene which normally functions to promote cellular proliferation can result in neoplastic transformation.

A number of studies have demonstrated amplification and overexpression of a number of cellular oncogenes (Biernat W, 1997; Brodeur et al., 1984; Fiddler et al., 1996; Garson et al., 1986; Khatib et al., 1993; Meddeb et al., 1996; Mitani et al., 1986; Reifenberger et al., 1994; Roberts et al., 1989; Wong et al., 1986). Since overexpression of a number of individual oncogenes inhibits myogenesis, we considered oncogenes present in the amplified region of the derivative 14 chromosome to be candidates for the inhibitory locus. We have determined that SAS, RARG1, VDR, CHOP, COL2A and MDM2 all to be amplified within the derivative 14 chromosome. All of the amplified genes were also expressed at elevated levels in the non-muscle forming hybrids. We determined, that in order to be classified as a candidate gene, overexpression of the gene of interest in C2C12 myoblasts should result in inhibition of muscle gene expression. Forced expression of VDR and CHOP both failed to affect muscle gene transcription in C2C12 cells. Analysis of the rhabdomyosarcoma cell line Rh30 revealed that it contained amplified CDK4, but not MDM2 (Reifenberger et al., 1994; Thayer, unpublished). Furthermore, transfer of the amplified CDK4 chromosome into C2C12 cells failed to inhibit muscle differentiation indicating that amplification of the CDK4 gene is insufficient to arrest the muscle program. MDM2 was demonstrated to interact with and inhibit the p53 tumor suppresser gene product (Haines et al., 1994; Kussie et al., 1996; Oliner et al., 1993). In addition, MDM2 was shown to bind the tumor suppresser pRb and the growth promoting E2F transcription factor (Xiao et al., 1995). Considering the above information pertaining to MDM2 and the other amplified and overexpressed genes, MDM2 became a primary candidate for inhibiting muscle gene expression in our microcell hybrid panel.

Evaluation and classification of the microcell hybrids was initially based on two criteria: 1) the ability to form mature myotubes following withdrawal of serum from the culture media, and 2) the detection of MHC *via* immunohistochemistry. To be classified as non-muscle, microcell hybrids need to show marked deficiencies of both criteria as compared to the C2C12 controls. Northern hybridization was employed to further characterize clones of interest. Paralleling the immunohistochemistry data, those clones that failed to produce normal amounts of MHC also demonstrated an inability to transcribe muscle-specific genes such as, MLC1/3 and Desmin. In order to determine the muscle gene transcription capacity of the microcell hybrids, we chose to assay for expression of two muscle reporter constructs: MCK-CAT and 4Rtk-CAT. The transcriptional control region of MCK-CAT is the upstream enhancer element that regulates transcription of the muscle creatine kinase gene (Weintraub et al., 1991). This complex 3300 base pair enhancer element contains multiple binding sites for transcription factors which include two MyoD binding sites. The 4Rtk-CAT construct is a much simpler construct and contains a tetrameric concatamer of the right MCK MyoD binding site as determined by *in vitro* binding studies (Blackwell, 1990; Davis et al., 1990; Lassar et al., 1989; Murre et al., 1989). All non-muscle forming microcell clones harboring the derivative 14 chromosome failed to activate transcription from either of these reporter constructs, while C2C12 cells initiated transcription from both constructs at levels 100 fold that found in the repressed clones. Activity from MCK-CAT, in the microcell hybrids, was slightly higher then that seen from 4Rtk-CAT. This is most likely do to the complex nature of the MCK enhancer element which allow for multiple factors to contribute to expression of the MCK-CAT construct. It is clear that expression from well-

defined muscle-specific control elements is repressed within those hybrids containing the derivative 14 and over-expressing MDM2.

To determine whether overexpression of MDM2 by itself was capable of extinguishing transcriptional activity from the muscle gene reporter constructs, we co-transfected MDM2 into C2C12 myoblasts along with the reporter cassettes and assayed MCK-CAT and 4Rtk-CAT activity. We demonstrate that MDM2 inhibits transcription from both MCK-CAT and 4Rtk-CAT, and has no effect on transcription from control vectors (Fiddler et al., 1996). These results indicate that MDM2 can repress gene transcription in a cell line that normally activates expression from these sequences under normal differentiation conditions. Because both reporter constructs contain binding sites for, and require functional members of the MyoD family to reach peak activity, one potential mechanism by which MDM2 can repress expression from these constructs would be by inactivating the myogenic bHLH proteins. To address this issue we assayed for transcriptional activity of the reporter constructs in 10T1/2 fibroblasts. 10T1/2 cells require exogenous MRF proteins in order to activate transcription from either MCK-CAT or 4Rtk-CAT. Co-transfections with MDM2 and either MyoD or myf-5 resulted in inhibition of transcription from the muscle reporter constructs when compared to assays lacking MDM2 expression. Therefore, MDM2 appeared to inhibit the transcriptional activation function of the MyoD family of proteins. To further complement the idea that MDM2 acts to suppress muscle gene transcription *via* inhibition of myogenic bHLH activity, overexpression of either MyoD or myf-5 rescues the muscle gene expression in the non-muscle hybrids (Fiddler, unpublished).

Our observations pertaining to overexpression of MDM2 in the non-muscle hybrids correlated with the ability of forced MDM2 expression to repress muscle gene transcription in C2C12 cells. To test whether MDM2 is responsible for extinguishing the muscle program in the microcell hybrids we constructed an anti-sense MDM2 vector and introduced it into non-muscle forming hybrids. Expression of anti-sense RNA is predicted to either bind mRNA molecules resulting in a non-translatable double stranded RNA complex or to simply stimulate the degradation of the double stranded species. The net result of either mechanism would be a reduction in the cellular concentration of the protein of interest. Given that the MDM2 half-life is approximately 20 minutes (Wu et al., 1993), we predicted that reduction of translatable MDM2 mRNA should markedly depress cellular levels of MDM2 protein. Transfection of anti-sense MDM2 resulted in a substantial increase in muscle gene transcription when assayed by expression from the reporter constructs MCK-CAT and 4Rtk-CAT.

Given that we demonstrated repression of muscle gene transcription by MDM2 occurs through the MyoD family, the potential of direct protein–protein interaction between the two proteins existed. MDM2 has been shown to bind p53 and repress transcription from p53 dependent reporter constructs (Lundgren K et al., 1997; Oliner et al., 1993). To test this hypothesis we employed the yeast two-hybrid system, and co-immunoprecipiations. None of the above techniques detected interaction between MyoD and MDM2. These experiments suggested that a direct interaction between MyoD and MDM2 does not occur either *in vitro* or *in vivo*. Although a direct interaction by which MDM2 extinguishes MyoD function was not shown, the potential of indirect interaction between MDM2 and some other protein required for MyoD function was intriguing.

Characterization of the MyoD protein revealed the existence of several domains that govern distinct functions (Davis, 1992; Thayer et al., 1989; Yutzey et al., 1990). We where interested in identifying a region and the specific function which was inhibited by MDM2. Electrophoretic mobility shift assays demonstrated that MyoD was capable of binding its consensus DNA site from non-muscle forming hybrid nuclear extracts (Fiddler, unpublished). Since MyoD binding to DNA did not appear altered in the presence of MDM2, the MyoD transactivation function became a suspect site through which MDM2 repression occurs. Experiments employing a MyoD-VP16 fusion protein and MyoD deletion mutants fused to the Gal DNA binding domain indicated that MDM2 does not repress the MyoD activation domain. Interestingly, we found MDM2 repression of MyoD to be DNA site specific. No transcriptional repression was noted on the ability of a GAL-MyoD fusion protein to activate a GAL-CAT construct while marked inhibition was observed on both MCK-CAT and 4Rtk-CAT. The basic region of MyoD has been established to be accountable for DNA site-specific binding, specifically amino acids 114 and 115 (Davis, 1992). Interestingly, MEF-2C was recently demonstrated to interact with MyoD through these two amino acids (Molkentin et al., 1995). This suggested that MDM2 may be extinguishing MyoD transcriptional activity by means of inactivating a co-factor required for MyoD function.

Recent studies demonstrated that cofactors exist and in some instances are required for MyoD function (Eckner et al., 1996; Puri Pieri, 1997; Sartorelli, 1997; Yuan, 1996). The presence of the p300 adapter protein was shown to be a requirement for MyoD mediated transcription. Likewise, MEF-2C has the potential to act as a cofactor for myogenic bHLH proteins and synergize E-box dependent transcriptional activity

(Molkentin et al., 1995). Although neither p300 nor MEF-2C is capable of activating the muscle program singularly, both appear to be required for myogenesis to take place in culture. Forced expression of p300 and the related CBP failed to reactivate muscle-specific gene transcription in the non-muscle forming microcell clones. Alternatively, expression of MEF-2C rescued muscle specific transcription in these clones indicating that the MDM2 inhibition is functioning through MEF-2C. Furthermore, data from the Thayer laboratory demonstrated that MDM2 associated with MEF-2C *in vitro* and that the protein interaction required the MEF-2C MADS domain and MDM2 amino acids 220-491.

MDM2 has been illustrated to bind to p53, pRb, and E2F (Haines et al., 1994; Martin et al., 1995; Xiao et al., 1995). It has been postulated that MDM2 exerts its growth promoting activity via binding to these proteins and either inactivating them, as with p53 and pRb, or activating their function, E2F. We have demonstrated, using p53 null cell lines, that MDM2 can effectively inhibit muscle gene expression in the absence of the p53 protein. In addition, forced expression of wild-type p53 failed to rescue muscle gene expression in the non-muscle forming hybrids. Therefore it seems unlikely that the muscle inhibitory effects of MDM2 are functioning through a p53 pathway. Furthermore, it has been demonstrated that the tumorgenic properties of MDM2 do not require the p53-binding domain (Sisalas et al., 1996). While addressing whether increases in E2F activity could contribute to repressing muscle differentiation, we found that forced expression of E2F in both C2C12 cells and 10T1/2 cell resulted in profound apoptosis. To address this indirectly, p130 an E2F inactivating gene was expressed in non-muscle forming hybrids. No enhancement of muscle gene transcription was noted in cells over expressing p130,

indicating that MDM2 is suppressing the muscle program in a manner distinct from that of activating E2F dependent transcription.

Interestingly, we found that muscle gene expression was elevated in the presence of high levels of MDM2 when cells were co-transfected with pRb. This enhanced transcriptional activity in the presence of excess MDM2 was seen in the non-muscle forming hybrids, and C2C12 cells transfected with the MDM2 expression construct. The rescue achieved in the microcell hybrids by expression of pRb, was never complete when compared to transcription from the same reporter constructs in C2C12 cells or forced expression of anti-sense MDM2. We found the expression of both p107 and pRb individually actually enhanced muscle gene transcription in C2C12 with wild-type levels of MDM2. This may be due to inactivation of any naturally occurring MDM2 protein, or that these pocket proteins function in some manner to promote muscle differentiation. The Nadal-Ginard laboratory has demonstrated pRb to be required for MyoD suppression of growth and myogenic activity (Gu et al., 1993). Additional data from this laboratory suggests that pRb and MyoD associate *in vitro* and *in vivo*. It was hypothesized that the interaction between pRb and MyoD may act to stabilize the MyoD-E2 DNA complex. Alternatively, loss of pRb in muscle culture results in cells that continue to synthesize DNA in spite of achieving myotube formation and proliferative arrest (Lassar et al., 1994; Novitch et al., 1996). The loss of pRb function in retinal cell culture results in the failure to terminally differentiate and may play a similar role in the muscle lineages.

It therefore appears that MDM2 functions through two distinct pathways, pRb and MEF-2C, in its ability to repress muscle gene transcription. Evidence from other laboratories indicates that MEF-2C plays an important role in regulating muscle development

(Molkentin et al., 1995; Olson et al., 1995). Our data suggests that inhibition of the synergistic role provided by MEF-2C with the myogenic bHLH family profoundly effects the transcription of muscle specific genes in a negative manner. Given that the MEF2 family is expressed in a wide variety of tissues, it seems reasonable to assume that these proteins may have synergistic functions in other cell lineages and that inhibition of their activity may result in deficiencies in differentiation. MDM2 over-expression has been demonstrated in a wide array of tumor types. One possible mechanism by which MDM2 promotes neoplastic growth would be through inhibition of the MEF2 family members. Since the target site for MDM2 interaction with MEF-2C is located in the MADS region, the potential for MDM2 interaction with members of the MADS family exists and could prove to be another avenue through which MDM2 can effect cellular differentiation.

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Appendix I

Primer	Primer Sequence	Target
Mp531-F	AACAGTGGCGGTCCACTTAC	Mouse p53 exon1
Mp531-R	AAGTCCCAATCCCAGCAAC	Mouse p53 exon1
PI-F	TCTGTCTTGCAGGACAATGC	Human PI
PI-R	ATTATTTCAGTGGAACGGCG	Human PI
MpRb-F	AGCTGCAATCCCCATTAATG	Mouse pRb
MpRb-R	AGGGTTGTTTTTTCGTGGC	Mouse pRb
ME2F-F	AACTGGGCAGCTGAGGTG	Mouse E2F1
ME2F-R	GAGTCCTCCGAAAGCAGTTG	Mouse E2F1
Alu5'	GTGAGCCACCGCGCCCGGCC	Inter-Alu
Alu3'	ACAGAGCGAGACTCCGTCTC	Inter-Alu
517	CGACCTCGACATCTYRGCTCACTGCAA	Inter-Alu
Rk712	GGCTCGCAAAAAGTCATCAT	Human RAP1B
Rk711	CCACCACAGGAAAGTCCATT	Human RAP1B
Rk911	GTTTCTTCTTACACTTCTTAC	Human SP1
Rk918	TCACAGAATGGTCTTCCTCT	Human Sp1
689	GTGTTCAAGAAGGAAGTATCTTC	Human DDIT3
988	ACAAGCTGAGACCTTTCCTTTTG	Human DDIT3
CDK2-F	CTGCTTATTAACACAGAGGGGG	Human CDK2
CDK2-R	GGAAACTTGGCTTGTAATCAGG	Human CDK2
CCND2-F	AGTGCGAAGAAGAGGTCTTCC	Human cyclin2
CCND2-R	TCCTCATCCTGCTGGAGC	Human cyclin2
BTG1-F	CTGTTCAGGCTTCTCCCAAG	Human BTG1
BTG1-R	TTGTGCCAGATCTTCACAGC	Human BTG1
Treb36-F	TTCGGATCTACCTGGGAGG	Human Treb36
Treb36-R	CAATGGCAATGTACTGTCCG	Human Treb36
SAS-F	AGTCCTTGGGACCACTTGG	Human SAS
SAS-R	GAAGATGAAGACCAAACCAAGG	Human SAS
cDNA31-F	CTGGGGTCAACAGTCCAGAT	cDNA3
cDNA31-R	GGGAAGGTACCATGTTGGG	cDNA3
ERBB3-F	CTGGGTGGATGAATTATGGG	Human erbb3
ERBB3-R	GGGGTGTGTGTGTGTGAGAG	Human erbb3
Tel-F	CACCATTGAACTGTTGCACC	Human Tel
Tel-R	GTTCATGTAAGCCAGGTCTTCC	Human Tel

Primer	Primer Sequence	Target
RK1121	ATCAAGACCATTGAGACCCG	Human PRPH
RK1120	CTTTGGATCATAGAGCCCCA	Human PRPH
RK710	AGGCTTCAATCTTGCTTCTCC	Human HOXC5
RK713	GATGGATCTGGAGGTGAGGA	Human HOXC5
RK709	GGTGAAAGGGACAGATAGATGC	Human RAR gamma
RK714	TGAGATGGAGGGCAGGAG	Human RAR gamma
NP1	AGCAGAGCGAGTAACTCACACAGTAG	Human NP
NP2	GTTGAGGAGCGAGAGGCAGTTATT	Human NP
PI1	GAAGCTCTCCAAGGCCGTGCATAA	Human PI
PI2	GTTGAGGAGCGAGAGGCAGTTATT	Human PI
TGFβ3A	AGACACATGGACAAGAATCCAGA	Human TGFβ3
TGFβ3B	GGTGAGCCTGAGCTTGCTCAAGAT	Human TGFβ3
FOS1	AGATTGCCAACCTGCTGAAGGAGA	Human Fos
FOS2	AGTCAGAGGAGGCTCATTGCTGC	Human Fos
H5314pst-1	CGTGCCCACAGGTCTACC	Clone H5313-4A
H5314pst-2	AGGGTAAAGGTCACGGGG	Clone H5313-4A
RK706	AGGTTCTATACCGAGCTTCCC	Human lactalbumin
RK707	GCTGGATTGGTTGGACAAGT	Human lactalbumin
CDK4-F	CATGTAGACCAGGACCTAAGG	Human CDK4
CDK4-R	GGAGGTCGGTACCAGAGTG	Human CDK4
R1MDM2	GCGCGAATTCAAACAAATGTGCAATAC CAACATG	MDM2 R1 Site introduced
SH88	GAAATTCGCCCGGAATT	Vector sequence
T3	AATTAACCCTCACTAAAGGG	Vector sequence
SK	CGCTCTAGAACTAGTGGATC	Vector sequence
KS	TCGAGGTCGACGGTATC	Vector sequence
T7	GTAATACGACTCACTATAGGGC	Vector sequence
MEF5'-1	AAAAGGATCCGGTCGAATTCCGAACGA	MEF2C BamHI site introduced
D14S274	Research Genetics, Inc. Primer Sets	Chromosome 14
D14S63	Research Genetics, Inc. Primer Sets	Chromosome 14
D14S255	Research Genetics, Inc. Primer Sets	Chromosome 14
D14S254	Research Genetics, Inc. Primer Sets	Chromosome 14
D14S277	Research Genetics, Inc. Primer Sets	Chromosome 14

Primer	Primer Sequence	Target
D14S67	Research Genetics, Inc. Primer Sets	Chromosome 14
D14S72	Research Genetics, Inc. Primer Sets	Chromosome 14
D14S78	Research Genetics, Inc. Primer Sets	Chromosome 14
D14S80	Research Genetics, Inc. Primer Sets	Chromosome 14
D14S34	Research Genetics, Inc. Primer Sets	Chromosome 14
D12S85	Research Genetics, Inc. Primer Sets	Chromosome 12
D12S82	Research Genetics, Inc. Primer Sets	Chromosome 12
D12S84	Research Genetics, Inc. Primer Sets	Chromosome 12
D12S106	Research Genetics, Inc. Primer Sets	Chromosome 12
D12S101	Research Genetics, Inc. Primer Sets	Chromosome 12
D12S81	Research Genetics, Inc. Primer Sets	Chromosome 12
D12S87	Research Genetics, Inc. Primer Sets	Chromosome 12
D12S58	Research Genetics, Inc. Primer Sets	Chromosome 12
D12S345	Research Genetics, Inc. Primer Sets	Chromosome 12
D12S64	Research Genetics, Inc. Primer Sets	Chromosome 12
D12S92	Research Genetics, Inc. Primer Sets	Chromosome 12
D12S95	Research Genetics, Inc. Primer Sets	Chromosome 12
D12S96	Research Genetics, Inc. Primer Sets	Chromosome 12
D12S313	Research Genetics, Inc. Primer Sets	Chromosome 12
D12S75	Research Genetics, Inc. Primer Sets	Chromosome 12