Activation of Osteoblast-like Phenotype in Microcell Hybrids and Characterization of MDM2 Functions in a Rhabdomyosarcoma

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CHAPTER 1

Activation of the Osteoblast-like Phenotype in C3H10T1/2 X Primary Fibroblast Cell Hybrids

Abstract

During early development, multipotential stem cells become committed to specific paths. Osteoblasts, the precursor cells for bone formation, are thought to derive from undifferentiated mesenchymal cells in the limb bud. According to morphological and biochemical changes during the cell differentiation, the developmental sequence of bone formation has been divided into three consecutive phases: proliferation, extracellular matrix maturation, and mineralization. Each phase involves the expression of a characteristic set of genes. Production of type I collagen takes place during proliferation of osteoblast precursor cells. The expression of alkaline phosphatase characteristically starts immediately after cession of cell proliferation, reaches a maximum during the phase of matrix maturation and declines as matrix mineralization commences. The genes expressed during matrix mineralization are those for the calcium-binding proteins, such as osteocalcin, osteonectin and osteopontin. Among these, only osteocalcin is a specific marker for osteoblast differentiation.

The mouse embryonic fibroblast C3H10T1/2 (10T1/2) is an established mesenchymal stem cell line which can differentiate into muscle, fat and cartilage cells upon the treatment with the demethylating agent 5-azadeoxycytidine (5-azaC). Our experiments showed that transferring human fibroblast chromosomes from primary cells into 10T1/2 cells by microcell mediated chromosome transfer, 10T1/2 cells are converted to osteoblast-like cells. These hybrids express the marker genes typical for osteogenesis.

Previous studies indicate that basic helix-loop-helix (bHLH) proteins may be involved in the regulation of osteogenesis. First, expression of Id, an antagonist of bHLH protein binding to the promoter, is down-regulated during osteoblast differentiation and overexpression of Id inhibits osteoblast differentiation; second, the promoter regions of several genes expressed in osteoblasts contain E boxes, which can act as DNA-binding domains for bHLH proteins; third, induction of osteoblastic differentiation is associated with increased binding of nuclear extracts to E boxes. I used a yeast two-hybrid screen to

identify the potential bHLH factors involved in the regulation of osteoblastic differentiation. From the screening, I pulled out two bHLH proteins dHAND (also called Th2) and Twist. They are expressed in the rat osteoblastic cell line ROS17/2 at both the proliferation and differentiation stage.

Introduction

1. Bone and its development

Bone is a specialized connective tissue that makes up together with cartilage, the skeletal system. These tissues serve three functions: (1) mechanical: support and site of muscle attachment for locomotion; (2) protective: for vital organs and bone marrow; and (3) metabolic: reserve of ions for the entire organism, especially calcium and phosphate.

The fundamental constituents of bone, as in all connective tissues, are the cells and the extracellular matrix. The latter is particularly abundant in this tissue and composed of collagen fibers and noncollagenous proteins. The matrix of bone has the unique ability to become calcified.

Anatomically, the skeleton is made up by two types of bones, flat bones (skull bones, scapula, mandible, and ileum) and long bones (tibia, femur, and humerus), which derive from two distinct types of histogenesis (intramemberanous and endochondral, respectively). The development and growth of long bones involves both types.

Descriptive embryology and anatomy distinguish two types of bone development: intramembranous and endochondral. Intramembranous ossification occurs when mesenchymal precursor cells differentiate directly into bone-forming osteoblasts, a process employed in generating the flat bones of the skull as well as in adding new bone to the outer surfaces of long bones. In contrast, endochondral bone formation entails the conversion of an initial cartilage template into the bone and is responsible for generating most bones of the skeleton. Cartilage templates originally form during embryogenesis when mesenchymal cells condense and differentiate into chondrocytes. These cells subsequently undergo a program of hypertrophy, calcification, and cell death. Concomitant neovascularization occurs, and osteoblasts and osteoclasts are recruited to replace the cartilage scaffold gradually with bone matrix and to excavate the bone marrow cavity. Longitudinal bone growth takes place through a similar pattern of endochondral ossification

in the growth plates located at the epiphyses (ends) of long bones. In these epiphyseal plates, the calcified, hypertrophic cartilage provides a scaffold for the formation of new trabecular bone. Ultimately, all remaining cartilage is replaced by bone except at the articular surfaces of the joints(reviewed in Eriebacher et al., 1995).

Three distinct embryonic lineages contribute to the early skeleton. The neural crest gives rise to the branchial arch derivatives of the craniofacial skeleton, the sclerotome generates most of the axial skeleton, and the lateral plate mesoderm forms the appendicular skeleton (reviewed in Eriebacher et al., 1995). The morphogenesis of the skeleton derives from the regulated differentiation, function, and interactions of its component cell types. Three major cell types contribute to the skeleton: chondrocytes, which form cartilage; osteoblasts, which deposit bone matrix; and osteoclasts, which resorb bone. Chondrocytes and osteoblasts are of mesenchymal origin, whereas osteoclasts derive from the hematopoietic system. Once embedded in bone matrix, osteoblasts mature into terminally differentiated osteocytes. The activity and differentiation of osteoblasts and osteoclasts are closely coordinated during development as bone undergoes continuous remodeling. More specifically, the formation of internal bone structures and bone remodeling result from coupling bone resorption by activated osteoclasts with subsequent deposition of new matrix by osteoblasts (reviewed in Eriebacher et al., 1995)

2. Osteoblast

Osteoblast is the precursor bone forming cells. Three forms of the osteoblast cell lineage are recognized. They are progenitor osteoblasts (preosteoblasts), mature osteoblasts, and osteocytes, which is an osteoblast that has become encased in calcified bone. The osteoblast is the bone lining cell responsible for the production of the matrix constituents (collagen and ground substance). It originates from a local mesenchymal stem cell, bone marrow stromal stem cell or connective tissue mesenchymal stem cell. These precursors, upon the right stimulation, undergo proliferation and differentiate into

preosteoblasts and then mature osteoblasts. A number of sequential steps are involved in the formation of bone. They are (1) synthesis and intracellular processing of type I collagen; (2) secretion and extracellular processing of the collagen; (3) the formation of microfibrils, fibrils, and ultimately fibers from the collagen; and (4) maturation of the collagen matrix with subsequent nucleation and growth of hydroxyapatite crystals. All of these functions are under the control of the osteoblast, with the eventual product being a fully calcified bone consisting by weight of 35% organic matrix and 65% inorganic crystalline material. Osteoblasts never appear or function individually, but are always found in clusters of cuboidal cells along the bone surface (~ 100-400 cells per bone forming site). Osteoblasts are always found lining a layer of bone matrix that they are producing and is not yet calcified (osteoid tissue). The presence of the osteoid is due to a time lag between matrix formation and subsequent calcification (Osteoid Maturation Period ~10 days). Behind the osteoblast are usually found one or two layers of cells, activated mesenchymal cells and preosteoblasts (reviewed in Baron, 1993).

The osteoblast is a very metabolically active cell and has an extensive rough endoplasmic reticulum producing collagen for the extracellular matrix. Positioned between the nucleus and rough endoplasmic reticulum is an intricate Golgi apparatus. In the cells, a substantial amount of the enzyme, alkaline phosphatase is present in the plasma membrane (reviewed in Puzas, 1993).

3. Bone matrix proteins and the mineralization process

The basic building block of the bone matrix fiber network is the type I collagen molecule, which is a triple-helical, coiled coil (supercoil) containing two identical a1(I) chains and a structurally similar but genetically different a2(I) chain. The collagen fibers are usually oriented in a preferential direction. Bone contains little, if any, of the other form of collagen found in the body and differs from other connective tissues in this regard. Type I collagen is by far the major collagenous component of bone and is believed to be the only

form necessary for true intramembranous bone formation (Eyre et al., 1988; Robins and Duncan, 1987; Termine, 1993).

Noncollagenous proteins (NCPs) comprise 10-15% of the total bone protein content. These can be broken down into four general groups of protein products: (1) cell attachment proteins; (2) proteoglycans; (3) γ-carboxylated (gla) proteins; and (4) growth-related proteins (Table 1) (Termine, 1993).

Table 1. Principal bone cell noncollagen products secreted to the bone matrix

Name	Approximate size	Potential function
Thrombospondin	450,000 (trimer)	Cell attachment
Fibronectin	440,000 (dimer)	Cell attachment, spreading
Biglycan (proteoglycan I)	170,000 (monomer)	Unknown
Decorin (proteoglycan II)	120,000 (monomer)	Collagen fibrillogenesis
Bone sialoprotein	75,000 (monomer)	Cell attachment, others unknown
Osteopontin	50,000 (monomer)	Cell attachment, spreading
Osteonectin	35,000 (monomer)	Ca^{2+}, mineralbinding; others unknown
Matrix gla protein	9,000 (monomer)	unknown
Osteocalcin	6,000 (monomer)	Ca^{2+} binding, bone turnover

All connective tissues interact with their extracellular environment in response to chemical stimuli that direct and/or coordinate specific cell functions, such as proliferation, migration, and differentiation. These particular interactions involve cell attachment and spreading via transient, focal adhesions to extracellular macromolecules. This is done via the intergrin family of cell surface receptors that transduce signals to the cytoskeleton

(Ruoslahti and Pierschbacher, 1987). Bone cells synthesize four proteins that affect cell attachment: fibronectin (FN), thrombospondin (TSP), osteopontin (OP), and bone sialoprotein (BSP) (Gehron Robey et al., 1988; Somerman et al., 1988). TSP, OP, and BSP are strong binders of ionic calcium and are found in the mineralized bone extracellular space (Fisher et al., 1987; Gehron Robey et al., 1988). OP is a reasonably well-phosphorylated phosphoprotein that, like FN and TSP, is found in bone and nonbone tissue systems (Mark et al., 1988).

Proteoglycans are macromolecules that contain acidic polysaccharide side chains (glycosaminoglycans) attached to a central core protein. In bone, two types of glycosaminoglycan are found: chondroitin sulfate, a polymer of sulfated N-acetylglucosamine and glucutonic acid, and heparin sulfate, a polymer of sulfated N-acetylglucosamine and glucuronic acid. The bone cell heparin sulfate proteoglycan product is membrane-associated and, as for all connective tissues, probably facilitates interaction of the osteoblast with extracellular macromolecules and heparin-binding growth factors (Hook et al., 1986; Beresord, 1987).

Chondroitin sulfate in bone is attached to three separate core proteins (Beresford et al., 1987). One of these is presumed to be pericellular in location and resembles a proteoglycan product synthesized by fibroblasts called versican (Krusius et al., 1987). Its role is not understood yet. The vast bulk of the glycosaminoglycans of bone are attached to two proteoglycan proteins that are similar but separate gene products (Fisher et al., 1987). These are called PG-I (which has two attached 50 KD chondroitin sulfate chains) and PG-II (having one attached chain). The functions of these proteins are generally assumed to be important for the integrity of most connective tissue matrices. They may also influence cell proliferation and differentiation in a variety of connective tissues including bone by their ability to bind and inactivate the transforming growth factor-β (TGF-β) family in the extracellular space (Okuda et al., 1990).

Vitamin K-dependent γ-carboxylation occurs on two bone NCPs, osteocalcin (bone gla-protein) and matrix-gla-protein (MGP). Osteocalcin represents one of the most abundant noncollagenous bone proteins and so far the only one that is exclusively expressed in bone (Carlson et al., 1993), whereas MGP is also found in cartilage. The synthesis of osteocalcin in bone is greatly stimulated by 1, 25$(OH)_2$ vitamin D_3, and its protein is now thought to be involved as a signal in the bone turnover cascade (Glowacki and Lian, 1985).

Other bone cell products may be associated with the growth and/or differentiation of the osteoblast in an indirect or as yet undefined fashion. One of the hallmarks of the osteoblast phenotype is the synthesis of high levels of alkaline phosphatase (Jukkola et al., 1993). This enzyme is associated with the plasma membrane of the osteoblast, which can bud out to form the matrix vesicles seen in developing bone. The function of this enzyme is not known with certainty. It may be involved in extracellular breakdown of pyrophosphate, a potent inhibitor of calcium phosphate deposition (Risteli and Risteli, 1993). The most abundant NCP produced by bone is osteonectin, a phosphorylated glycoprotein accounting for ~2% of the total protein of developing bone in most animal species. The protein has high affinity for binding ionic calcium and physiological hydroxyapatite (Termine et al., 1981). It also binds to collagen (Termine et al., 1981) and thrombospondin (Clezardin et al., 1988). Osteonectin is found in platelets (Stenner et al., 1986) and in nonbone tissues that are rapidly proliferating, remodeling, or undergoing profound changes in tissue architecture (Holland et al., 1987; Wewer et al., 1988). Thus the protein is associated with growing tissue, and in nonbone systems. Its transcription and synthesis appear to be shut down or absent under steady-state conditions.

A number of proteins in bone appear to be associated with the life cycle and function of the osteoblast. These proteins consist of growth factors, such as TGF-β1-5 and insulin-like growth factors, osteoblast secretion products that can stimulate osteoblast cell

growth in an autocrine and/or paracrine fashion (Robey et al., 1987) (Canalis et al., 1988). Thus, the growth potential of a bone may result from its own genetic framework and involve transcription of both known growth factors and their receptors in the same cell population.

4. Gene expression during osteoblast differentiation

During development, multipotential stem cells progressively become committed to specific paths. This process, called determination or commitment, irreversibly alters the cells so that the progeny will inherit a new and now more limited potential. Once committed or determined, the precursor cell may exist in this state until it differentiates. The study of bone and cartilage cell differentiation in vitro provides an important counterpart to the study of these cell populations in vivo. In vitro approaches have also made valuable contributions to our understanding of the differentiation of chondrocytes and osteoblasts. By analogy with other cell types, chondrocyte and osteoblast differentiation involve at least two distinct processes. First, precursor cells commit to the cartilage or bone lineages. Second, consecutive maturation events lead to a fully differentiated phenotype, a process required in vivo for the constant replenishment of cartilage and bone cells from their respective committed precursor cell populations (Eriebacher et al., 1995). The synthesis, deposition and mineralization of the organic matrix of bone involves the ordered expression of a number of genes by the osteoblasts. The developmental sequence of an osteoblastic cell phenotype has been divided into three consecutive phases: proliferation, extracellular matrix maturation, and mineralization (Jukkola et al., 1993). Each phase involves the expression of a characteristic set of genes and is a prerequisite for the next. Production of type I collagen is an early event, taking place during proliferation of osteoblast precursor cells. If deposition of a collagenous matrix by proliferating osteoblastic cells in vitro is disrupted, the cells do not enter the next developmental phase of the osteoblast phenotype (Jukkola et al., 1993). It is clear that a cell committed to the osteoblast lineage no longer

expresses type III collagen, which is otherwise always co-expressed with type I in mesenchymal cells; the regulatory events leading to this suppression are not known in detail. The expression of alkaline phosphatase characteristically starts immediately after cession of cell proliferation, reaches a maximum during the phase of matrix maturation and declines as matrix mineralization commences (Jukkola et al., 1993). Among the genes expressed during matrix mineralization are those for the calcium-binding proteins osteocalcin, osteopontin and osteonectin.

5. Transcriptional regulation of osteoblast differentiation

The process by which eukaryotic cells control the expression of their genes constitutes one of the key unanswered questions in biology. While it is clear that post-transcriptional and post-translational controls are involved in the fine tuning of gene expression, it seems that the primary level of control is exhibited at the transcriptional level. Condensed chromatin structures and DNA methylations act as repressors of structural genes in embryonic cells and in adult cell types (Groudine and Weintraub, 1992). One of the transcriptional controls involves the methylation of specific cytosine residues within and around genes (Razin and Riggs, 1980). Vertebrate DNAs contain 5-methylcytosine ($5^{m}C$) as a minor base, and modification of this site in the sequence CpG is consistent with the activities of expression of some genes. The methylation sites exist in three states: (1) sites which are never methylated, (2) those which are always methylated irrespective of the differentiated state of the cell, and (3) those which demonstrate tissue-specific variability in the degree of methylation (Mandel and Chambon, 1979). The relationship between DNA modification and gene expression is not an all-or-none situation.

Taylor and Jones (1979) first reported the striking observation that brief exposure of mouse C3H10T1/2 (10T1/2) fibroblasts to 5-azaC converts descendants into myocyte, chondrocyte, or adipocyte clones (Taylor and Jones, 1979). Conversion of 10T1/2 cells into these three mesenchymal cell lineages requires incorporation of 5-azaC into DNA

(Taylor and Jones, 1982). Incorporation of 5-azaC inhibits cellular methyltransferases (Jones and Taylor, 1980) and this results in the demethylation of CpG residues and the subsequent activation of specific genes. It is also likely that phenotypic conversion is a heritable trait. Analysis of colonies arising from individual cells treated with 5-azaC indicates that the probability of phenotypic conversion is quite high: 25-50% of the colonies contain myocytes, 7-28% contain adipocytes, and 1-7% contain chondrocytes (Konieczny and Emerson, 1984; Taylor and Jones, 1982). A small percentage (1-16%) of colonies contain multiple cell lineages.

Genomic DNA transfection experiments indicate that myoblast DNA, but not 10T1/2 DNA, converts 10T1/2 cells into stably determined myoblasts (Lassar et al., 1986). The frequency of myogenic conversion is consistent with the transfer of a single genetic locus, and supports the hypothesis that a myogenic determination gene that is inactive in 10T1/2 cells becomes structurally modified during conversion of 10T1/2 cells to the myoblast lineage following treatment with 5-azaC. The genomic transfection experiments also suggest that 10T1/2 cells do not express dominant *trans*-acting factors that can repress the transferred myogenic determination gene. Introduction of human fibroblast chromosome 11, containing the human muscle determination gene MyoD, into 10T1/2 cells results in activation of this gene and, consequently, activation of the muscle phenotype (Thayer and Weintraub, 1990). This result indicates that human MyoD is not repressed by a *cis* (methylation) mechanism in primary fibroblasts. In contrast to monochromosomal hybrids, whole-cell hybrids formed by fusing primary fibroblasts with 10T1/2 cells fail to activate MyoD expression. Furthermore, transfer of human chromosome 11 into 10T1/2 cells activates expression of MyoD, while transfer of chromosome 11 and an additional human chromosome, chromosome 4, did not result in activation of MyoD (Woloshin et al., 1995). This inhibition is mediated by a homobox gene, MSX1, expressed from human fibroblast chromosome 4, which interacts with an enhancer located upstream of the MyoD gene. If a similar phenomenon is occurring on the loci that control activation of the other

phenotype in 10T1/2 cells, it is likely that transfer of other human primary fibroblast chromosomes would result in activation of these phenotypes.

The mouse embryonic fibroblast cell line 10T1/2 is a mesenchymal progenitor cell line (Reznikoff et al., 1973). It can be converted to muscle, fat, and cartilage upon the treatment with 5-azaC. Transfection of 10T1/2 cells with BMP-2 and BMP-4 expression vectors induces differentiation into osteoblasts (Ahrens et al., 1993; Wang et al., 1993), so these cells have the potential to differentiate into osteoblasts. Since a previous study showed that transfer of human chromosome 11 (containing MyoD) into 10T1/2 cells turned on the muscle differentiation pathway in this cells, I propose that transfer of other human primary fibroblast chromosomes into 10T1/2 cells may result in activation of the osteoblast phenotype.

The control of the bone differentiation has been extensively studied. Members of the transforming growth factor-β (TGF-β) superfamily of secreted growth and differentiation factors have received considerable attention as potent regulators of mesenchymal differentiation in vivo and in vitro. Bone morphogenetic proteins (BMP), which belongs to the TGF-β superfamily, are thought to regulate the early commitment of mesenchymal cells to the chondrogenic and osteogenic lineages, as suggested by their ability to induce ectopic cartilage and bone formation at nonskeletal sites such as muscle (Wozney, 1992; Wozney et al., 1988). The BMPs comprise a growing family of more than 12 proteins, 9 of which have been shown individually to induce ectopic bone in an in vivo assay system. All but BMP-1 are members of the TGF-β superfamily (Riley et al., 1996). Among them, the functions of BMP-2 and BMP-4 have been intensively studied. They are present during embryonic development in a spatial and temporal pattern consistent with involvement in skeletogenesis (Riley et al., 1996). They induce bone and cartilage formation. BMP-2 induces the undifferentiated mesenchymal progenitors to differentiate into osteoblasts, chondrocytes and adipocytes (Ahrens et al., 1993; Wang et al., 1993).

They can also stimulate osteoblast differentiation of both uncommitted and committed mesenchymal cells in vitro (Vukicevic et al., 1989; Wozney, 1992; Yamaguchi et al., 1991) and can induce cells other than osteoblasts to express osteoblastic markers (Katagiri et al., 1994; Murry et al., 1993; Yamaguchi, 1995).

6. Involvement of basic helix-loop-helix proteins in osteoblast differentiation

Development and differentiation of cells are under the control of various classes of transcription factors which have been identified through genetic and biochemical means. One group of factors with pivotal roles during development are the basic helix-loop-helix (bHLH) proteins. bHLH proteins have been implicated in the determination and differentiation of several cell lineages: the MyoD subfamily in myogenesis, the *achaete scute* gene products and their mammalian homologues in neurogenesis, and the E-proteins in B-cell development (Jan and Jan, 1993; Weintraub, 1994; Zhuang et al., 1994). The bHLH motif contains a basic region followed by two amphipathic α-helices separated by a loop structure. The HLH region is important in mediating dimerization with another HLH sequence which in turn forms a functional bipartite DNA-binding domain from the two respective basic regions. The basic region binds to the major groove of a core DNA sequence, CANNTG, which is referred to as an E-box motif (Murre et al., 1989). This interaction is responsible for transcriptional activation of specific genes.

The bHLH proteins can be divided into several subclasses based on their expression patterns, DNA-binding ability, dimerization specificities, and transcriptional activity. The ubiquitously expressed Class A bHLHs, which includes E2A gene products (Murre et al., 1989), HEB (Hu et al., 1992), and the *Drosophila daughterless* gene product (Caudy et al., 1988). Class A bHLH proteins act as partners for at least two of the other classes. The Class B bHLH proteins are tissue-specific and include the MyoD family of myogenic regulatory factors (Weintraub, 1994), and the mammalian and *Drosophila*

achaete scute neurogenic factors (Jan and Jan, 1993). The Class B bHLH proteins heterodimerize with the Class A proteins to function as transcriptional activators (Weintraub et al., 1990). Another class of proteins, termed the dominant negative family of HLH proteins (dnHLH), lacks the region responsible for DNA binding but still maintains the HLH region necessary and sufficient for dimerization (Ellis et al., 1990). The dnHLH proteins, comprised of the Id family and the related *Drosophila emc* gene product, negatively regulate the transcriptional activity of bHLH proteins by preferentially binding to Class A bHLHs, preventing them from heterodimerizing with the tissue specific Class B bHLHs (Benezra et al., 1990; Sun et al., 1991; Ellis et al., 1990). Down regulation of the dnHLH proteins allows the Class A and Class B bHLH to efficiently heterodimerize to form functional transcriptional activators (Benezra et al., 1990). Evidence of this has been shown in vitro binding studies and has been proposed in the mutual exclusion of expression of tissue specific bHLH and dnHLH transcripts during mouse embryogenesis (Benezra et al., 1990) (Goldfard et al., 1996) (Wang et al., 1992) (Ellmeier and Weith, 1995). Hairy and Enhancer-of-split [h/E(Sp1)] bHLH proteins form the fourth class whose interactions with the other three groups are not well characterized (Ish-Horowicz and Pinchin, 1987) (Ishibashi et al., 1993) (Knust et al., 1987).

Although no osteoblast-specific bHLH proteins have yet been identified, indirect evidence suggest that bHLH proteins may be involved in upregulating differentiation and repressing proliferation in osteoblasts. With regard to differentiation, E-box binding domains are present in the osteocalcin, osteopontin, and alkaline phosphatase gene promoters, which are sequential markers of osteoblast differentiation (Siddhanti and Quarles, 1994) (Tamura and Noda, 1994). In addition, there is functional data linking bHLH proteins to the expression of the osteoblastic phenotype through E-box binding motifs in the osteocalcin promoter. By deletion and site-directed mutagenesis analysis of the rat osteocalcin promoter region, Tamura et al (Tamura and Noda, 1994) found that the E-box is involved in bHLH mediated expression of the gene in osteoblasts. Furthermore,

electrophoresis mobility shift assay (EMSA) experiments indicated the formation of osteoblast-specific protein/DNA complexes with an E-box sequence, and this binding activity was induced in 10T1/2 cells by BMP-2 treatment. Also, Id mRNA, which inhibits bHLH actions, is abundantly expressed in proliferating and undifferentiated MC3T3E1 preosteoblasts and decreases prior to osteoblast-specific gene expression (Ogata and Noda, 1991). This inverse relationship between Id expression and osteoblastic differentiation is consistent with Id involvement in osteoblastic differentiation. Moreover, overexpression of Id prevents expression of alkaline phosphatase in developing osteoblasts (Murray et al., 1992). In addition, various other non-tissue-specific regulatory bHLH proteins, including twist and E-proteins, have been identified in osteoblasts (Murray et al., 1992). Recent studies in ROS 17/2.8 osteosarcoma cells also support a role of HLH proteins in the control of osteoblastic-related gene expression. In ROS17/2.8 cells, vitamin D-mediated upregulation of osteocalcin is associated with a decrease in Id and increased binding of nuclear extracts to E-box motifs (Kawaguchi et al., 1992). bHLH proteins may also repress proliferation by inhibiting the transcription of early response genes. E-box cis-acting domains overlap the serum response element in the c-fos promoter. In non-osteoblastic systems, members of the bHLH family of proteins have been shown to act as negative regulators of c-fos transcription (and cellular proliferation) by blocking the transactivating function of the SRE (Trouche et al., 1992). This suggests that tissue-specific transcription factors in osteoblasts can repress proliferation.

7. Objectives

The primary goal of this study was trying to identify the factors that regulate osteoblast differentiation. It has been shown that the mouse mesenchymal cell line 10T1/2 can be converted to muscle, fat, and cartilage upon the treatment with 5-azaC (Taylor and Jones, 1979). Transfection of 10T1/2 cell with BMP-2 and BMP-4 expression vector induces it to differentiate into osteoblasts (Ahrens et al., 1993; Wang et al., 1993), so it has

the potential to differentiate into osteoblast upon the addition of the right set of trancriptional factors. Previous studies showed that transfer of human chromosome 11 (containing MyoD) into 10T1/2 cells turned on the muscle differentiation pathway in these cells (Thayer and Weintraub, 1990). I tested whether transfer of other human primary fibroblast chromosomes into 10T1/2 cells result in activation of the osteoblast phenotype.

bHLH proteins have been shown to regulate the differentiation of several tissues. The presence of bHLH proteins in osteoblasts and their apparent regulation by factors modulating differentiation suggest that osteoblast-specific bHLH family members may control the osteoblastic developmental program, I used a yeast two-hybrid screen to identify the potential bHLH factors involved in the regulation of osteoblastic differentiation.

Materials and Methods

1. Plasmids: LexA-fusion plasmid pBTM116, pVP16 and pLex-lamin were provided by Dr. Stan Hollenberg. pBTM116, the LexA DNA-binding domain fusion vector, contains the following components: (1) the TRP1 gene, a selectable marker for yeast, (2) the complete LexA coding sequence, with a downstream polylinker for cloning genes of interest to generate in-frame protein fusions to LexA, expressed from the yeast ADHI promoter, (3) a yeast 2μ origin of replication, and (4) a bacterial origin of replication and (β-lactamase gene, a selectable marker for E.Coli) (Bartel et al., 1993). pLex-Lamin is a derivative of pBTM-116 with the insertion of Lamin gene. pVP16, the VP16 activation domain fusion vector, contains the following components: (1) the LEU2 gene, a selectable marker for yeast and E.Coli, (2) the simianvirus 40 (SV40) large T antigen nuclear localization sequence (NLS) fused to the VP16 acidic activation domain, with a downstream polylinker for cloning genes of interest to generate in-frame protein fusions to VP16, expressed from ADHI promoter, (3) a yeast 2μ origin of replication, and (4) a bacterial origin of replication and β-lactamase gene (Hollenberg et al., 1995). The plasmids CSTh2, CSαsTh2(5'), CSαsTh2(3') were also provided by Dr. Stan Hollenberg. These plasmids contain the coding sequence of mouse Th2 (dHAND) and the 5' and 3' antisense sequence of Th2 individually. Plasmid pECE.Twi contains the coding sequence of mouse Twist which was provided by Dr. Barbara Wold. Plasmid pOC-CAT is the osteocalcin promoter driven CAT construct.

2. Yeast strains: yeast strains AMR70 and L40 were provided by Dr. Stan Hollenberg. Strain AMR70 is used for mating analysis. The genotype of it is: *Matα his3 lys2 trp1 leu2*

URA3::(lexAop)$_8$-lacZ GAL4; this genotype is incomplete. The genotype of L40 is: *Mata his3(200 trp1-901 leu2-3,112 ade2 LYS2::(lexAop)$_4$-HIS3 URA::(lexAop)$_8$-lacZ GAL4*; this genotype is incomplete (Hollenberg et al., 1995).

3. Cell lines: The mouse mesenchymal cell line C3H10T1/2 (10T1/2) (Reznikoff et al., 1973) (American Type Culture Collection) was used as recipient for microcell fusion (Thayer and Weintraub, 1990). ROS17/2 is a rat osteoblast-like osteosarcoma cell line (Rodan and Majeska). It was provided by Dr. Robin Leach. These lines were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Maryland) supplemented with 15% bovine calf serum. The donor cell line, Human skin fibroblast (HSF), was provided by Dr. R. E. K. Fournier. HSF and the microcell hybrids were maintained in DMEM supplemented with 15% Bovine calf serum and 250mg/ml Geneticin (G418) (Gibco BRL). The hybrids were plated out at clonal density grown for two weeks and allowed to differentiate for additional 10 days.

4. Rat osteoblast cDNA fusion library: Total RNA was prepared from rat osteoblast cell line ROS17/2 . To select poly(A)$^+$ RNA, the RNA was double purified by using the polyAT tract mRNA Isolation system II and III (Promega). The double-strand cDNA was synthesized from 50ng of RNA with 600 ng (~500pmol) of phosphorylated random hexamer by using the cDNA synthesis kit SuperScript™ choice system (Gibco BRL). The ends of the resulting cDNA were filled with T4 DNA polymerase and then *Pol* I (klenow). After enzyme inactivation and DNA precipitation, the cDNA was ligated overnight with a 500-fold molar excess of the *Not* I adaptor (5'-P-ATCCTCTTAGACTG CGGCCGCTCA-3' [NL-s] and 5'-P TGAGCGGCCGCAGTCTA AGAG-3' [NL-as]) (provided by Dr. Stan Hollenberg). cDNA (350-700nt) was purified by agarose gel electrophoresis. One-tenth of the size-fractionated cDNA was then PCR amplified with the NL-s primer; the

PCR buffer contained 5 mM Mg^{2+} and the cycle profile was 94°C for 30 sec, 62°C for 30 sec, and 72 °C for 2 min. To convert all of the cDNA to fully paired duplexes, a portion of the reaction was diluted 10-fold, fresh PCR reagents were then added, and a single cycle of amplification was performed. The cDNA was then digested overnight with *Not* I, repurified by agarose gel electrophoresis, and ligated into pVP16 (*Not* I, dephosphorylated).

5. Microcell-mediated chromosome transfer: The HSF donor cell line Human skin fibroblast was micronucleated by adding colcemid (Sigma) to a final concentration of 10mg/ml and incubated for 48 hours. The microcells were enucleated by centrifugation in 10mg/ml cytochalasin B (Sigma) (Fournier and Ruddle, 1977; McNeill and Brown, 1980). The microcells were resuspended in 100mg/ml phytohaemagglutinin-P (Difco) and added to 10T1/2 monolayer (60-70% confluence) for adhesion. The cells were fused by exposure to 50% PEG1540 (w/v) (Baker) solution in DMEM. Following fusion, they were passaged into complete medium DMEM containing HAT [pH9.5-9.7] (0.136% Hypoxanthine, 0.0019% Aninopterin, 0.0386% Thymidine, 0.0025% Phenol red) and 500mg/ml G418. Individual clones were isolated with cloning cylinders after two weeks of selection.

6. Alkaline Phosphatase staining: The differentiated cells were permealized in ice cold 50mg/ml lysolecithin (Sigma) solution followed by three washes with ice cold PBS. Then the cells were incubated with 0.33mg/ml Nitroblue tetrazolium (NBT) and 0.165mg/ml Bromochloroindoyl phosphate (BCIP)(Gibco BRL) in alkaline phosphatase buffer (100mM NaCl, 5mM $MgCl_2$, 100mM Tris [pH9.5]) for 30 minutes. The reaction was stopped by rinsing the cells with PBS containing 20mM EDTA. The positive cells were detected by the appearance of a dark purple precipitate by light microscopy.

7. Northern blot analysis: Cytoplasmic RNA from adherent tissue culture cells was isolated by lysing the cells in TSM (140mM NaCl, 10mM Tris [pH7.4], 1.5mM $MgCl_2$) plus 0.5% NP40 solution. After extraction with phenol: chloroform (1:1), the RNA was precipitated by adding 1/20 volume of 3M NaCl and 2 volumes of 100% ethanol at -20°C overnight. Ten micrograms of RNA were loaded per lane and electrophoresed in 1.2% agarose gels containing 6.7% formaldehyde. The RNA was transferred to GeneScreen Hybridization membrane (Dupont) by capillary transfer in 10XSSC (1.5M NaCl, 150mM sodium citrate). RNA was crosslinked by exposure to UV light followed by baking at 80°C for 2 hours. Blots were prehybridized in hybridization buffer (50% formamide, 1% bovine serum albumin [Fraction V], 1mM EDTA, 0.5M sodium phosphate [pH7.2]), 7% sodium dodecyl sulfate) for 1 hour at 42°C . ^{32}P-labeled probes were added to the hybridization buffer for 17-24 hours at 42°C. The blots were subsequently washed in 2XSSC plus 0.1% SDS at room temperature for 15 minutes. and 0.1XSSC, 0.1% SDS at 55°C for 30 minutes.

8. Karyotypic analysis: Slides of metaphase spreads were used for both G-banded preparations and Fluorescent in situ hybridization (FISH). Total genomic human DNA was nick translated using standard protocols to incorporate biotin-11-dUTP and used as probe. Hybridizations were carried out on slides at 37°C for 16 hours. Final probe concentrations varied from 40-60 ng/ml. Signal detection was carried out as described by Trask and Pinkel (Trask and Pinkel, 1990). Amplification of the biotinylated probes utilized alternate incubations of slides with antiavidin (Vector) and fluorescence isothiocyanate (FITC-Extravidin, Sigma). Amplification of digoxygenated probes utilized alternate incubations of slides with FITC-tagged sheep antibodies made in rabbits and FITC-tagged rabbit antibodies made in sheep (Boehringer Mannheim). Slides were stained with propidium iodide (0.3mg/ml) and viewed under UV fluorescence with FITC filters (Zeiss). Metaphase spreads were photographed with Fuji color film (ASA 100) at ASA 400. Identification of

human chromosomes were determined 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. Slides of metaphase spreads were also trypsin G-banded using standard protocols (Yunis and Chandler, 1978). A minimum of 20 cells were analyzed for the presence of human chromosomes. G-banded metaphase spreads were photographed with technical pan 2415 film (Kodak) at ASA 40.

9. Yeast Two-hybrid: The bait construct pLex-da, is the fusion of *Drosophila* daughterless bHLH domain with the LexA DNA binding domain (Hollenberg et al., 1995). One liter of L40, containing pLex-da, was transformed with 500 mg of rat osteoblast ROS17/2 cDNA library by the lithium method (Schiest and Gietz, 1989) with dimethyl sulfoxide addition (10% final (Hill et al., 1991)). After 8 hours recovery in one liter of yeast complete medium (Trp^- Leu^- Ura^-), the transformants were plated on medium to select for histidine prototrophy (Trp^- Leu^- Ura^- His^- Lys^-). Histidine positive colonies were lysed in liquid nitrogen and analyzed for β-galactosidase activity on filters. Positive colonies were further analyzed after loss of the Lex-da plasmid. pLex-da or pLex-lamin DNA was introduced by mating with AMR70 derivatives containing these plasmids. Library DNA from colonies which were negative with Lex-Lamin and positive with Lex-da was isolated by transformation into the bacterial strain MC1066 and by selection of leucine prototrophy on minimal plates. Sense and antisense primers used for automated sequencing were 5'-GAGTTTGAGCAGATGTTTA-3' and 5'-TGTAAAACGACGGCCAGT-3' respectively. Sequences in all six reading frames were tested against the National Center for Biotechnology Information nonredundant protein database by using the blastx search program.

10. **Cell transfections**: Cells were transiently transfected by the Lipofectamine method (Gibco BRL). Approximately 3 X 10^5 cells were plated 1 day prior to transfection into 60-mm-diameter tissue culture plates. On the day of transfection, the cells were washed twice with serum-free DMEM. Transfection mixtures consisted of a total DNA content of 1-4 μg. The lipid-DNA mixtures were added to the washed cells and brought to a final volume of 2 ml with serum-free DMEM. The transfection mixtures were allowed to remain on the cells for 6 hours, after which the DMEM containing 30% calf serum was added to the cells. The transfection solution was removed by aspiration the following day and the cells were washed twice with serum-free DMEM. Then the cells were refed with DMEM containing 15% calf serum and harvested after approximately 48-72 hours.

11. **CAT assays**: Chloramphenicol acetyltransferase (CAT) activity was measured by a phase extraction procedure (Scrable et al., 1990). In brief, after transfection, cell extracts were generated by freeze-thawing cell pellets in 100 μl of 0.25 M Tris (pH7.5). Following treatment at 65°C for 15 minutes to inactivate endogenous acetylases, 15 μl of extracts was assayed with 0.2 mCi of [^{3}H]chloramphenicol (Dupont-New England Nuclear) and 250 nM butyryl coenzyme A (Sigma) in a total volume of 100 μl. The reaction was allowed to proceed for 2 to 5 hours at 37°C and was stopped by mixing 200 μl of tetramethylpentadecane (TMPD)-xylene (2:1) (Sigma), and then 140 μl of the upper phase was added to the scintillation cocktail and the radioactivity was counted.

12. **Assay for β-Galactosidase in extracts of mammalian cells**: The procedure is done according to the instruction of the manual of *Molecular Cloning* (Sambrook, 1989). Briefly, mix 3 μl of 100 x Mg solution (0.1 M $MgCl_2$, 4.5 M β-mercaptoethanol, 66 μl 1

x ONPG (o-nitrophenyl-β-D-galactopyranoside) (Sigma)), 30 µl cell extract and 201 µl 0.1 M sodium phosphate (pH7.5) for each sample to be assayed. Incubate the reactions at 37°C for 30 minutes or until a faint yellow color has developed. Stop the reactions by adding 500 µl of 1 M Na_2CO_3 to each reaction. Read the optical density of the reactions at a wavelength of 420 nm.

Results

1. Microcell-mediated chromosome transfer

10T1/2 cells can be converted to muscle, fat and cartilage cell types (Taylor and Jones, 1979), and we previously observed conversion of 10T1/2 cells to muscle by introducing chromosome 11. Therefore, we tested a panel of 10T1/2 microcell hybrids for conversion to other phenotypes. In order to obtain a large number of 10T1/2 microcell hybrids retaining different human chromosomes, we generated a microcell hybrid panel in which random human fibroblast chromosomes, tagged with a neo^r retrovirus, were introduced by microcell fusion. This microcell hybrid panel contains 14 hybrids, with each hybrid retaining between one and ten human chromosomes (Thayer and Weintraub, 1990). We originally reported that 2 out of the 14 hybrids displayed a low level of myogenesis, and that both of these hybrids retained human chromosome 11, and therefore human MyoD. Visual inspection of the remaining hybrids indicated that three of them, 10HSm-6, 10HSm-14 and 10HSm-15, exhibited a distinct phenotype when these cells reached high density (Figure 1). The phenotype of these hybrids is different from their parental fibroblasts: the cells rounded up, accumulated large cytoplasmic vacuoles and grew to form multilayered structures. Among these three hybrids, clone 10HSm-15 exhibited the strongest phenotypic change.

2. Phenotypic characterization of the hybrids

To determine the phenotype of these hybrids, I did a series of assays on them. Because previous studies showed that 10T1/2 cells have the capacity to differentiate into fat, cartilage and bone (Taylor and Jones, 1979; Wang et al., 1993), I tested differentiation specific markers for each of these phenotype. Each hybrid clone was assayed for expression of the adipocyte phenotype by staining colonies from each hybrid with oil red-O, which identifies cells containing lipid deposits. None of the three hybrids showed

positive staining for the fat phenotype. To test for activation of the cartilage phenotype, each hybrid clone was stained with Alcian blue. All three hybrids, 10HSm-6, 10HSm-14, and 10HSm-15 were stained positively with Alcian blue (data not shown). Because Alcian blue stains acidic mucopolysaccharides and does not assay expression of specific gene products. I next use Northern blot hybridization to assay for expression of type II and X collagens, which are specific markers for cartilage differentiation (Castagnoia et al., 1988). Surprisingly, 10HSm-6, -14, and -15 did not express these two collagens (data not shown). Thus, it seems likely that these hybrids do not express the chondrocyte phenotype. However, since osteoblasts express a related phenotype, secretion of an acidic mucopolysaccharide matrix and expression of alkaline phosphatase, I assayed these hybrids for expression of alkaline phosphatase and other bone markers. First, I used histochemical staining to detect the expression of alkaline phosphatase. The hybrid 10HSm-15 was plated out at clonal density and allowed to differentiate for 14 days. The cells were stained for the presence of alkaline phosphatase enzyme activity. It was detected in the extracellular region of the differentiated cells (Figure 2), especially those that grew to multilayers. I further tested these cells with other osteoblast differentiation markers. Northern analysis of RNA isolated from hybrid 10HSm-15 grown under differentiation conditions demonstrated that both the hybrid 10HSm-15 and the parental cell line 10T1/2 expressed osteonectin and osteopontin, but only the hybrid expressed the osteoblast-specific marker osteocalcin (Figure 3). I conclude that transferring human chromosomes from primary fibroblast into mouse mesenchymal cell line 10T1/2 can induce a phenotype change of 10T1/2 from fibroblast into osteoblast-like cells.

3. Characterization of the chromosomes responsible for the phenotypic change

In order to determine which chromosomes were responsible for the phenotypic change, extensive karyotypic analysis was performed on hybrids 10HSm-6, 10HSm-14

and 10HSm-15. Cells were analyzed using both Trypsin G-banding and FISH with total genomic human DNA followed by R-banding of metaphase spreads. All these hybrids retained multiple chromosomes and human chromosomes 6, 8, 14 and 15 were present in high frequency in almost every cell counted (Figure 4). From this results, I speculated that a gene on one of these chromosomes is responsible for the phenotype change. I transferred each chromosome into 10T1/2 cells by microcell fusion and failed to obtain clones having the same phenotype observed in the multiple chromosome hybrids. This may imply that multiple loci, which are on different chromosomes, are responsible for the osteoblast differentiation.

4. Expression of basic helix-loop-helix proteins in the osteoblastic cell line ROS17/2

Because several studies indicated the presence of bHLH factors in the differentiation of osteoblasts (Siddhanti and Quarles, 1994) (Tamura and Noda, 1994) (Ogata and Noda, 1991) (Murray et al., 1992) (Kawaguchi et al., 1992), I set up a yeast two-hybrid screen to identify the bHLH proteins expressed in the osteoblast cell line ROS17/2. The bait used was the *Drosophila* counterpart of mammalian E proteins, daughterless (da). It has an interaction specificity very similar to that of E47 and homodimeric association of da was not detectable (Hollenberg et al., 1995). Thus, da was used to screen the random-primed ROS17/2 cDNA library for interacting proteins. The results are summarized in figure 4. A total of 1.2×10^7 yeast cells were transformed with a cDNA fusion library, and about 1254 clones were capable of growing in the absence of histidine. One-third of these clones were β-galactosidase positive. These positive yeast cells were streaked onto adenine plates to remove the original bait plasmid. The cells that lose the bait turned pink. These cells were mated with the yeast strain AMR70 containing the plasmid pLex-Lamin. The majority of the colonies were also positive with Lex-Lamin (Bartel et al., 1993) suggesting non-specific pairing with Lex-da or direct reporter

activation in the absence of a LexA fusion. The plasmids of the Lex-Lamin-negative colonies were recovered and digested by four base enzyme cutters to identify clones bearing the same inserts. Sixty three clones were sequenced. The sequences revealed five distinct HLH proteins and some HLH associated proteins. The five HLH proteins included three members of the Id family: Id1, Id2, and Id3. Among them, Id2 was present in high frequency. The other two bHLH factors are: twist (Wolf et al., 1991) and dHAND (Th2) (Srivastava et al., 1995) (Figure 5).

By northern blot hybridization, I found that both twist and dHAND (Th2) are expressed in proliferating and differentiated osteoblasts (Figure 6), but only basal levels were detected in the 10T1/2 cells. Since the promoter region of osteocalcin has three E-boxes (one CACATG and two CAGCTG which are located within 300bp of the transcription start site) (Murray et al., 1992), the expression of osteocalcin may be regulated by bHLH proteins. In order to determine whether the expression of osteocalcin is regulated by twist and/or dHAND in the osteoblast differentiation pathway, I tested their regulations of the osteocalcin promoter region by CAT assay. The reporter construct that I used is called pOC-CAT which is the 840 bp osteocalcin promoter region cloned into the pCAT-Basic vector.

Antisense RNA is an effective tool for silencing gene expression, and can result in partial or complete knockout of gene function. To determine the function of dHAND (Th2) on the regulation of the osteocalcin promoter region, I tested whether expression of antisense Th2 (dHAND) would affect the activity of pOC-CAT in the ROS17/2 cells. One of the antisense construct of Th2 contains the 5' region of the Th2 coding sequence which includes the bHLH region. The another one contains the 3' region of Th2 coding sequence. These constructs were cloned in the antisense orientation in the expression vector pCS2+ which contains the simian CMV IE94 promoter/enhancer. They were provided by Dr. Stanley Hollernberg. From my CAT assay result, I did not observe a specific regulation

activity of Th2 (dHAND) on the promoter region of osteocalcin comparing with its regulation on the control plasmid RSV-βgal (Figure 7).

The same effect was also observed with the transfection assay done with the Twist expression vector. By transfecting the Twist expression vector pECE.Twi with the reporter construct pOC-CAT into the 10T1/2 cells, the result did not show a specific effect of this bHLH factor on the regulation of the osteocalcin promoter region (Figure 8).

From my results, it shows that these two bHLH factors may not regulate the osteocalcin promoter region directly and they may be involved in the regulation of other genes that expressed during the osteoblast differentiation pathway. Another possibility is that they may need cofactors that are absent in the cells that I assayed. Because the osteocalcin promoter region that I used is only 840 bp which may not contain the regions that that these two factors bind to. The role of these bHLH proteins on the differentiation of the osteoblast need to be further investigated.

Discussion and Conclusion

1. Regulation of osteoblast differentiation

The development and remodeling of bone requires the differentiation of mature osteoblastic cells from undifferentiated proliferating mesenchymal osteoprogenitor cells. Furthermore, the decline in osteoblastic cell number and function that occurs with aging and in pathologic processes results, at least in part, from deficiencies in this mechanism. The roles of hormones, growth factors, and other agents in the regulation of this process have been studied extensively in animals, organ culture, and in vitro osteoblastic cell lines. However, the precise biochemical and molecular mechanisms that execute the induction of the differentiated osteoblastic phenotype are unknown. In other tissues, nuclear regulatory molecules play pivotal roles in the induction of differentiation. Great progress has been made recently in understanding the molecular mechanisms governing the differentiation process in skeletal muscle, which, like bone, is derived from embryonic mesoderm. Taylor and Jones (1979) showed that brief exposure of the mouse embryonic fibroblast cell line 10T1/2 to 5-azaC is sufficient to generate colonies capable of forming muscle, fat, and cartilage. This conversion is thought to occur by demethylation and subsequent expression of specific loci involved in determining each cell type (Jones and Taylor, 1980). Genomic DNA transfection experiments indicate that myoblast DNA, but not 10T1/2 DNA, converts 10T1/2 cells into stably determined myoblasts (Lassar et al., 1986). Later, it was shown that introduction of human fibroblast chromosome 11, containing human MyoD, into 10T1/2 cells results in activation of MyoD and, consequently, the muscle phenotype (Thayer and Weintraub, 1990). This result indicates that human MyoD is not repressed by a *cis* (methylation) mechanism in primary fibroblasts, and that the 10T1/2 MyoD gene is likely repressed by methylation. If a similar phenomenon is occurring on the loci that control activation of other committed phenotypes in 10T1/2 cells, it seems likely that

transfer of other human primary fibroblast chromosomes would result in activation of other phenotypes.

In this study, I utilized the microcell mediated chromosome transfer technique to randomly transfer human fibroblast chromosomes into 10T1/2 cells. In general, hybrid cells formed by fusing dissimilar cell types fail to express the tissue specific products of either parent, a phenomenon known as extinction (Davison, 1974). Extinction of particular traits is mediated by discrete, trans-dominant, genetic loci (Killary and Fournier, 1984). Extinction is not an irreversible change: hybrid segregants that have lost extinguisher loci reexpress previously extinguished traits (Killary and Fournier, 1984; Weiss and Chaplain, 1971). In addition, activation of previously silent tissue-specific products encoded by heterologous parental genomes has been observed. Activation has been largely restricted to hybrids in which gene dosage is strongly biased (Brown and Weiss, 1975; Darlington et al., 1982) or when chromosome segregation has occurred (Killary and Fournier, 1984; Peterson et al., 1985). Therefore, activation usually occurs when there is an absence of extinction.

Our results show that transfer of human fibroblast chromosomes into mouse 10T1/2 cells can convert these cells into osteoblast-like cells. When the cells became confluent, they round up, accumulate large cytoplasmic vacuoles and form multilayered structures. The differentiated cells expressed the osteoblastic differentiation markers, alkaline phosphatase, osteopontin (Mark et al., 1987), osteonectin (Termine et al., 1981), and osteocalcin (Carlson et al., 1993), which I determined either by histochemical staining or northern blotting analysis. Although the parental 10T1/2 cells also expressed the alkaline phosphatase, osteopontin and osteonectin at a basal level, 10T1/2 cells do not express the osteoblast specific gene, osteocalcin, which is expressed at the mineralization stage of bone differentiation. Karyotypic analysis of these three hybrids indicated that they retain four human chromosomes in common, 6, 8, 14, and 15. Like the expression of MyoD in 10T1/2 cells, the conversion of fibroblast into osteoblast suggests that there is one

or a set of genes on these chromosomes that is (are) responsible for activation of the osteoblast pathway in 10T1/2 cells. It is unlikely that the human chromosomes bearing these osteoblast markers were transferred into the 10T1/2 cells. Transferring all the chromosomes containing these markers into a single cell by microcell fusion is very unlikely, because these genes reside on different chromosomes : alkaline phosphatase and osteocalcin are on chromosome 1, osteonectin is on chromosome 5, and osteopontin is on chromosome 4. From our karyotypic analysis results, these chromosomes do not present in the hybrids. Therefore, the expression of these genes in the hybrids is due to the activation of the differentiation factors.

The activation mechanism of the potential osteoblast determination factor (OsteoD) may share a similar pathway with those myogenic determination factors. OsteoD may activate its downstream determination genes which subsequently activate the bone structure genes, and then turn on the bone differentiating cascades. Like MyoD, human OsteoD may be properly regulated in murine cells, and activation of the human OsteoD gene in 10T1/2 cells is not due to species differences.

Since hybrids 6, 14, 15 share the chromosome 6, 8, 14 and 15 in common, identification of the chromosome(s) responsible for this conversion can be addressed by transferring each chromosome, individually or in combination, into 10T1/2 cells and inspecting the hybrids for the bone phenotype. I have transferred neo^r markered chromosome 6, 8, 14 and 15 into 10T1/2 cells individually, but failed to observe the osteoblast-like phenotype of the initial hybrids by visual inspection. One reason for this phenomenon may be that: since chromosome fragmentation can occur in microcell hybrids, the individual chromosome hybrids that I obtained may contain different fragments instead of intact chromosomes. Therefore, I simply missed the one that contained the correct gene. This is unlikely since I scored at least twenty hybrids for each chromosome. Another explanation may come from the phenomenon observed by Taylor and Jones (Taylor and Jones, 1982). Analysis of the 10T1/2 cell colonies treated with 5-azacytidine for their

probability of phenotypic conversion: 25%-50% of the colonies contain myocytes, 7%-28% contain adipocytes, and 1%-7% contain chondrocytes, a small percentage (1%-16%) contain multiple cell lineages. The high incidence of myocyte colonies suggests that undermethylation of one or a few loci is sufficient to convert the 10T1/2 cell into the myogenic lineage (Konieczny and Emerson, 1984) and transfer of this unmethylated locus into 10T1/2 cells would convert the cells into myocytes. This was proved by transfection of chromosome bearing the MyoD gene into the 10T/2 cells indeed turned on the muscle differentiation pathway in these cells (Lassar et al., 1986; Thayer and Weintraud, 1990). Because the conversion of adipocytes and chondrocytes is two and seven fold lower than myocytes and they did not really observe the osteocytes in these 5-azacytidine treated colonies, the activation of these phenotypes may involve multiple loci. Unmethylation or transfer of one unmethylated locus is not sufficient to turn on the differentiation pathway. It may require transferring multiple loci which may reside on different chromosomes into the 10T1/2 cells. This can be addressed by transferring different combinations of chromosomes into 10T1/2 cells by microcell fusion.

2. Expression of bHLH proteins in osteoblast cell line

Previous studies indicate that basic helix-loop-helix proteins may be involved in the regulation of osteogenesis. First, expression of Id, an antagonist of bHLH protein binding to the promoter, is down-regulated during osteoblast differentiation and overexpression of Id inhibits osteoblast differentiation (Kawaguchi et al., 1992; Ogata and Noda, 1991); second, the promoter regions of several genes expressed in osteoblasts contain E boxes, which can act as DNA-binding domains for bHLH proteins (Siddhanti and Quarles, 1994); third, induction of osteoblastic differentiation is associated with increased binding of nuclear extracts to E boxes (Kawaguchi et al., 1992). Though the presence of bHLH proteins in osteoblasts and their apparent regulation by factors modulating differentiation suggest that osteoblast-specific bHLH family members may control the osteoblastic

development, the specific bHLH proteins need to be defined in osteoblasts and characterized in the transcriptional control of osteoblast differentiation. In this study, I used a modified version of the two-hybrid system which has been used to search for tissue-specific bHLH transcription factors (Hollenberg et al., 1995). A widely expressed bHLH protein, Drosophila E protein *daughterless*, was used as a binding partner. bHLH proteins have been efficiently isolated with this system. I used this bait to screen an osteoblast cell line ROS17/2 cDNA library and five bHLH proteins were identified in this screen. Three of them, Id1, Id2, Id3, belong to the universally expressed dominant negative Id family. The other two, Twist and dHAND, are recently identified bHLH proteins. They are expressed in both the proliferating and differentiated ROS17/2 cells. The three Id proteins may negatively regulate the function of tissue-specific bHLH in the cells as suggested by their gene structure and other studies (Benezra et al., 1990; Murray et al., 1992; Sun et al., 1991).

The other two bHLH proteins, twist and dHAND, are fairly new members of the bHLH family. dHAND is expressed in the bilateral heart primordia and subsequently throughout the primitive tubular heart and its derivatives during chick and mouse embryogenesis. It may play roles in the regulation of the morphogenetic events of vertebrate heart development (Srivastava et al., 1995). twist was originally cloned in *Drosophila.* It is both essential for the establishment of mesodermal cell fate before gastrulation and is involved in subdividing the mesoderm later in development, especially for myogenesis (Thisse et al., 1988; Baylies and Bate, 1996). The mouse twist homologue also is expressed in a dynamic pattern (Wolf et al., 1991). It is initially found throughout the somatic mesoderm but subsequently is excluded from the myotome, persisting only in dermomyotomal and sclerotomal cells. Unlike its homologue in *Drosophila*, M-twist acts as an inhibitor of muscle differentiation and this inhibition is reversible (Hebrok et al., 1994). M-twist inhibits muscle differentiation by two mechanisms: it prevents trans-activation of muscle target genes by MEF2 and it dimerizes with the E proteins, thereby sequestering

them from forming functional heterodimers with myogenic factors (Spicer et al., 1996). twist is expressed in scleroderm during development and it has been shown that treatment of osteoblastic cells MC-3T3-E1 with Doxorubicin (an antineoplastic agent) inhibits differentiation of the cells and enhances the M-twist expression in both growth and differentiation conditions (Glackin et al., 1992).

I tested the function of these two proteins on the promoter region of osteocalcin which has three bHLH DNA-binding sites (one CACATG and two CAGCTG). By CAT assay, I did not observe significant effect of these proteins on the osteocalcin promoter region. I speculate that they may not interact with the osteocalcin promoter directly or they regulate the osteoblast differentiation pathway with other partner proteins. By themselves, they have minimal effect on the expression of the bone structural genes. Because the promoter region of osteocalcin that I used is only the 840 bp region that is closed to the transcription start site, the DNA binding regions for Twist and dHAND may be absent. Also, the antisense constructs that I used may not block the functional region of Th2 specifically. Therefore, I did not observe an effect of this protein on the osteocalcin promoter region. Further experiments need to be conducted to determine how these bHLH factors regulate the osteoblast differentiation pathway.

3. Conclusion

In conclusion, I showed that transfer of a specific set of primary fibroblast chromosomes into 10T1/2 cells results in activation of the osteoblast phenotype. This indicates that a specific set of genes exist which are responsible for the osteoblast differentiation. Microcell mediated chromosome transfer should be useful to the identification of these genes. Also, our yeast two-hybrid screening told us that there may be tissue specific bHLH proteins expressed in the osteoblast cells. Further studies need to be carried out to determine their functional involvement in osteoblast differentiation.

Once specific factors are identified in bone differentiation, studies addressing the interaction between these transcriptional regulators and known secreted osteoinductive factors will be of great importance in understanding the patterning and regulation of mesenchymal differentiation in vivo. Understanding the molecular control of osteoblast function and its relationship to external stimuli and intracellular signaling systems is fundamental to understanding how these cells differentiate and proliferate. Such information may permit the development of pharmacological approaches to selectively manipulate the pre-osteoblast replication, the differentiation process and mature osteoblast function. The ability to manipulate these components of the osteoblast developmental sequence may allow separate induction of coupled and de novo bone formation, which would be useful for treatment of a variety of human disease states characterized by osteopenia or overabundant osseous production.

Reference

Ahrens, M., Ankenbauer, T., Schroder, D., Hollnagel, A., Mayer, H., and Gross, G. (1993). Expression of human bone morphogenetic proteind-2 or -4 in murine mesenchymal progenitor C3H10T1/2 cells induced differentiation into distinct mesenchymal cell lineages. DNA and Cell Biol. *12*, 871-880.

Baron, R. (1993). Anatomy and Ultrastructure of Bone. Primers on the metabolic bone disease and disorders of mineral metabolism *1*, 3-9.

Bartel, P., Chien, R., Sternglanz, R., and Fields, S. (1993). Using the two-hybrid system to detect protein-protein interactions. In D. A. Hartley (ed), Cellular interactions in development: a practical approach. IRL Press, Oxford, 153-179.

Baylies, M. K., and Bate, M. (1996). *twist*: a myogenic switch in *Drosophila*. Science *272*, 1481-1484.

Benezra, R., Davis, R. L., Lockshon, D., Turner, D. L., and Weintraub, H. (1990). The protein Id: A negative regulatory helix-loop-helix DNA binding protein. Cell *61*, 49-59.

Beresford, J. N., Fedarko, N. S., Fisher, L. W., Midura, R. J., Yanagishita, M., Termine, J. D., and Gehron Robey, P. (1987). Analysis of the proteoglycans synthesized by human bone cells *in vitro*. J Biol Chem *262*, 17164-17172.

Brown, J. E., and Weiss, M. C. (1975). Activation of production of mouse liver enzymes in rat hepatoma-mouse lymphoid cell hybrids. Cell *6*, 481-494.
Canalis, E., McCarthy, T., and Centrella, M. (1988). Isolation and characterization of insulin-like growth factor I (somatomedin-C) from cultures of fetal rat calvariae. Endocrinology *122*, 22-27.

Carlson, C. S., Tulli, H. M., Jayo, M. J., Loeser, R. F., Tracy, R. P., Mann, K. B., and Adama, M. R. (1993). Immunolocation of noncollagenous bone matrix proteins in lumbar vertebrate from intact and surgically menopausal cynomolgus monkeys. J. Bone Min. Res. *8*, 71-81.

Castagnoia, P., Dozin, B., Moro, G., and Cancedda, R. (1988). Changes in the expression of collagen genes show two stages in chondrocyte differentiation in vitro. The Journal of Cell Biology *106*, 461-467.

Caudy, M., Vassin, H., Brand, M., Tuma, R., Jan, L. Y., and Jan, Y. N. (1988). *daughterless*, a *Drosophila* gene essential for both neurogenesis and sex determination, has sequence similarities to *myc* and the achaete-scute complex. Cell *55*, 1061-1067.

Clezardin, P., Malaval, L., Ehrensperger, A. S., Delmas, P., Dechavanne, M., and McGregor, J. L. (1988). Complex formation of human thrombospondin with osteonectin. Eur J Biochemistry *175*, 275-284.

Darlington, G. J., Rankin, J. K., and Schlanger, G. (1982). Expression of human hepatic genes in somatic cell hybrids. Somatic Cell Genetics *8*, 403-412.

Davison, R. L. (1974). Gene expression in somatic cell hybrids. Annual Review Genetics *8*, 195-218.

Ellis, H. M., Spann, D. R., and Posakony, J. W. (1990). extramachrochaete, a negative regulator of sensory organ development in Drosophila, defines a new class of helix-loop-helix proteins. Cell *61*, 27-38.

Ellmeier, W., and Weith, A. (1995). Expression of the helix-loop-helix gene Id3 during murine embryonic development. Dev. Dyn. *203*, 163-173.

Eriebacher, A., Filvaroff, E. H., Gitelman, S. E., and Derynck, R. (1995). Toward a Molecular Understanding of Skeletal Development. Cell *Vol. 80*, 371-378.

Eyre, D. R., Dickson, I. R., and Van Ness, K. (1988). Collagen cross-linking in human bone and articular cartilage, Age-related changes in the content of mature hydroxypyridinium residues. Biochem. J. *252*, 495-500.

Fisher, L. W., Hawkins, G. R., Tuross, N., and Termine, J. D. (1987). Purification and partial characterization of small proteoglycans I and II, bone sialoproteins I and II and osteonectin from the mineral compartment of developing human bone. J. Biol. Chem. *262*, 9702-9708.

Fournier, R. E. K., and Ruddle, F. H. (1977). Microcell-mediated transfer of murine chromosomes into mouse, Chinese hamster, and human somatic cells. Proc. Natl. Acad. Sci. *USA 74*, 319-323.

Gehron Robey, P., Young, M. F., Fisher, L. W., and McClain, T. D. (1988). Thrombospondin is an osteoblast-derived component of mineralized extracellular matrix. J. Cell Biol. *108*, 719-727.

Glackin, C., Murray, E. J. B., and Murray, S. S. (1992). Doxorubicin inhibits differentiation and enhances expression of the helix-loop-helix genes Id and mTwi in mouse osteoblastic cells. Biochemistry International *28*, 67-75.

Glowacki, J., and Lian, J. B. (1985). Impaired recruitment of osteoclast progenitors by osteocalcin deficient bone implants. In: Butker WT (ed) The Chemistry and Biology of Mineralized Tissues. Ebsco Media. Birmingham, 164-169.

Goldfard, A. N., Lewandowska, K., and Shoham, M. (1996). Determinants of helix-loop-helix dimerization affinities. J. Biol. Chem. *271*, 2683-2688.

Groudine, M., and Weintraub, H. (1992). Propagation of globin DNAase I-hypersensitive sites in the absence of factors required for induction : a possible mechanism for determination. Cell 30: 131-139. Cell *30*, 131-139.

Hebrok, M., Wertz, K., and Fuchtbauer, E. M. (1994). M-twist is an inhibitor of muscle differentiation. Developmental Biology *165*, 537-544.

Hill, J., Donald, K. A., and Griffiths, D. E. (1991). DMSO-enhanced whole cell yeast transformation. Nucleic Acid Research *19*, 5791.

Holland, P. W. H., Harper, S. J., McVey, J. H., and Hogan, B. L. M. (1987). *In vivo* expression of mRNA for the ca^{2+}-binding protein SPARC (osteonectin) revealed by *in situ hybridization*. J. cell biology *105*, 473-482.

Hollenberg, S. M., Sternglanz, R., Cheng, P. F., and Weintraub, H. (1995). Identification of a new family of tissue-specific helix-loop-helix proteins with a two-hybrid

system. Mol. Cell. Biol. *15*, 3813-3822.

Hook, M., Woods, A., Johansson, S., Kjellen, L., and Couchman, J. (1986). Functions of proteoglycans at the cell surface. In: Evered E, Whelan J (eds) Functions of the proteoglycans, Ciba Foundation Symposium 124. John Wiley and Sons, Chichester, 143-156.

Hu, J. S., Olson, E. N., and Kingston, R. E. (1992). HEB, a helix-loop-helix protein related to E2A and ITF2 that can modulate the DNA-binding affinity of myogenic regulatory factors. Mol. Cell. Biol. *12*, 1031-1042.

Ish-Horowicz, D., and Pinchin, S. M. (1987). Pattern abnormalities induced by ectopic expression of the Drosophila gene Hairy are associated with repression of ftz transcription. Cell *51*, 405-415.

Ishibashi, M. Y., Sasau, S., Nakanishi, S., and Kageyama, R. (1993). Molecular characterization of HES-2, a mammalian helix-loop-helix factor structurally related to Drosophila hairy and Enhancer of split. European J. Biochem. *215*, 645-652.

Jan, Y. N., and Jan, L. Y. (1993). HLH proteins, fly neurogenesis and veterbrate myogenesis. Cell *75*.

Jones, P. A., and Taylor, S. M. (1980). Celluar differentiation, cytidine analogs and DNA methylation. Cell *20*, 85-93.

Jukkola, A., Risteli, L., Melkko, J., and Risteli, J. (1993). Procollagen synthesis and extracelluar matrix deposition in MG-63 osteosarcoma cells. J Bone Miner. Res, in press.

Yamaguchi, A., Komaki, M., Abe, E., Takahashi, N., Ikeda, T., Rosen, V., Wozney, J. M., Fujisawa-Sehara, A., and Suda, T. (1994). Bone morphogenetic Protein-2 Converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage. J. Cell Biol. *127*, 1755-1766.

Kawaguchi, N., DeLuca, H. F., and Noda, M. (1992). Id expression and its suppression by 1, 25-dihydroxyvitamin D3 in rat osteoblastic osteosarcoma cells. Proc. Natl. Acad. Sci. USA *89*, 4569-4572.

Killary, A. M., and Fournier, R. E. K. (1984). A genetic analysis of extinction: Trans-Dominant loci regulate expression of liver-specific traits in hepatoma hybrid cells. Cell *38*, 791-800.

Knust, E., Bremer, K. A., Vassin, H., Ziemer, A., Tepass, U., and Campos-Ortega, J. A. (1987). The enhancer of split locus and neurogenesis in Drosophila melanogaster. Dev. Biol. *122*, 262-273.

Konieczny, S. F., and Emerson, C. P. (1984). 5-azacytidine induction of stable mesodermal stem cell lineages from 10T1/2 cells: evidence for regulatory genes controlling determination. Cell *38*, 791-800.

Krusius, T., Gehlsen, K. R., and Ruoslahti, E. (1987). A fibroblast chondroitin sulfate proteoglycan core protein contains lectin-like and growth factor-like sequences. J. Biol. Chem. *262*, 17164-17172.

Lassar, A. B., Paterson, B. M., and Weintraub, H. (1986). Transfection of a DNA locus that mediates the conversion of 10T1/2 fibroblasts to myoblasts. Cell *47*, 649-56.

Mandel, J. L., and Chambon, P. (1979). DNA methylation: Organ specific variations in the methylation pattern within and around ovalbumin and other chicken genes. Nucleic Acids Research *7*, 2081-2103.

Mark, M. P., Prince, C. W., Gay, S., Austin, R. L., Bhown, M., Finkelman, R. D., and Butler, W. T. (1987). A comparative immunocytochemical study on the subcelluar distribution of 44 kDa phosphoprotein and bone gamma-carboxyglutamic (gla)-containing protein in osteoblasts. J. Bone Min. Res. *2*, 337-346.

Mark, M. P., Prince, C. W., Gay, S., Austin, R. L., and Butler, W. T. (1988). 44kDal bone phophoprotein (osteopontin) antigenicity at ectopic sites in newborn rats: kidney and nervous tissues. Cell Tissue Research *251*, 23-40.

McNeill, C. A., and Brown, R. L. (1980). Genetic manipulation by means of microcell-mediated transfer of normal human chromosomes into recipient mouse cells. Proc. Natl. Acad. Sci. USA *77*, 5394-5398.

Murray, S. S., Glackin, C. A., Winters, K. A., Gazit, A., Kahn, A. J., and Murray, E. J. (1992). Expression of helix-loop-helix regulatory genes during differentiation of mouse osteoblastic cells. J. of Bone and Min. Res. *7*, 1131-1138.

Murre, C., McCaw, P. S., and Baltimore, D. (1989). A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and myc proteins. Cell *56*, 777-783.

Murry, S. S., Murry, E. J. B., Glackin, C. A., and Urist, M. R. (1993). Bone morphogenetic protein inhibits differentiation and affects expression of Helix-Loop-Helix regulatory molecules in myoblastic cells. J. Cell. Biochem. *53*, 51-60.

Ogata, T., and Noda, M. (1991). Expression of Id, a negative regulator of helix-loop-helix DNA binding proteins, is down-regulated at confluence and enhanced by dexamethasone in a mouse osteoblastic cell line, MC3T3E1. Biochemical and Biophysical Research Communications *180*, 1194-1199.

Okuda, S., Languino, L. R., Ruoslahti, E., and Border, W. A. (1990). Elevated expression of transforming growth factor-b and proteoglycan production in experimental glomerulonephritis. J. Histochem. Cytochem. *38*, 1549-1563.

Peterson, T. C., Killary, A. M., and Fournier, R. E. K. (1985). Chromosome assignment and trans regulation of the tyrosine aminotransferase structural gene in hepatoma hybrid cells. Mol. Cell. Biol. *5*, 2491-2494.

Puzas, J. E. (1993). The osteoblast. Primers on the metabolic bone disease and disorders of mineral metabolism. *1*, 15-21.

Razin, A., and Riggs, A. D. (1980). DNA methylation and gene function. Science *210*, 604-610.

Reznikoff, C. A., Brankow, D. W., and Heidelberger, C. (1973). Establishment and characterization of a cloned line of C3H mouse embryo cells sensitive to postconfluence inhibition of division. Cancer Research *33*, 3231-3238.

Riley, E. H., Lane, J. M., Urist, M. R., Lyons, K. M., and Lieberman, J. R. (1996). Bone Morphogenetic Protein-2. Clinical Orthopedics and Related Research., 39-46.

Risteli, L., and Risteli, J. (1993). Biochemical Markers of Bone Metabolism. Annuals of Medicine *25*, 385-393.

Robey, P. G., Young, M. F., Flanders, K. C., Roche, N. S., Kondaiah, P., Reddi, A. H., Termine, J. D., Sporn, M. B., and Roberts, A. B. (1987). Osteoblasts synthesize and respond to transforming growth factor-type β (TGF-β) in vitro. J. of Cell Biol. *105*, 457-63.

Robins, S. P., and Duncan, A. (1987). Pyridinium cross-links of bone collagen and location in peptides isolated from rat femur. BioChem. Biophys. Aca. *914*, 233-239.

Rodan, G. A., and Majeska, R.-J. in limb development and regeneration, eds. Kelly, R. D., Goetick, P. F. & MacCabe, J. A. (liss, New York), Part B, 249-259.

Ruoslahti, E., and Pierschbacher, M. D. (1987). New perspective in cell adhesion. Science *238*, 491-497.

Schiest, R. H., and Gietz, R. D. (1989). High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. Current Genetics *16*, 339-346.

Scrable, H. J., Johnson, D. K., Rinchik, E. M., and Cavenee, W. K. (1990). Rhabdomyosarcoma-associated locus and MYOD1 are syntenic but separate loci on the short arm of human chromosome 11. Proc. Natl. Acad. Sci. USA *87*, 2182-2186.

Siddhanti, S. R., and Quarles, L. D. (1994). Molecular to pharmacologic control of osteoblast proliferation and differentiation. J. of Cell. Biochem. *55*, 310-320.
Somerman, M. J., Fisher, L. W., Foster, R. A., and Sauk, J. J. (1988). Human bone sialoprotein I and II enhance fibroblast attachment *in vitro*. Calcif. Tissue Int. *43*, 50-53.

Spicer, D. B., Rhee, J., Cheung, W., and Lassar, A. (1996). Inhibition of Myogenic bHLH and MEF2 transcription factors by the bHLH protein Twist. Science *272*, 1476-1480.

Srivastava, D., Cserjesi, P., and Olson, E. N. (1995). A subclass of bHLH proteins required for cardiac morphogenesis. Science *270*, 1995-1999.

Stenner, D. D., Tracy, R. P., Riggs, B. L., and Mann, K. G. (1986). Human platelets contain and secrete osteonectin, a major protein of mineralized bone. Proc. Natl. Acad. Sci. USA *83*, 6892-6896.

Sun, X.-H., Copeland, N. G., Jenkins, N. A., and Maltimore, D. (1991). Id proteins Id1 and Id2 selectively inhibit DNA binding by one class of helix-loop-helix proteins. Mol. Cell. Biol. *11*, 5603-5611.

Tamura, M., and Noda, M. (1994). Identification of a DNA sequence involved in osteoblast-specific gene expression via interaction with helix-loop-helix (HLH)-type transcription factors. J. of Cell Biol. *126*, 773-782.

Taylor, S. M., and Jones, P. A. (1982). Changes in phenotypic expression in embryonic and adults cells treated with 5-azacytidine. J. Cell Physiology *111*, 187-194.

Taylor, S. M., and Jones, P. A. (1979). Multiple new phenotypes induced in 10T1/2 and 3T3 cells treated with 5-azacytidine. Cell *17*, 771-9.
Termine, J. D. (1993). Bone Matrix Proteins and the Mineralization Process. Primers on the metabolic bone disease and disorders of mineral metabolism, 21-25.

Termine, J. D., Kleinmann, H. K., Whitson, S. W., Conn, K. M., McGarvey, M. L., and Martin, G. R. (1981). Osteonectin, a bone specific protein linking mineral to collagen. Cell *26*, 99-105.

Thayer, M. J., and Weintraud, H. (1990). Activation and repression of Myogenesis in somatic cell hybrids: Evidence for trans-negative regulation of MyoD in primary fibroblasts. Cell *63*, 23-32.

Thisse, B., Stoetzel, C., Gorostiza-Thisse, C., and Perrin-Schmitt, F. (1988). Sequence of the *twist* gene and nuclear localization of its protein in endomesodermal cells of early *Drosophila* embryos. The EMBO J. *7*, 2175-2183.

Trask, B., and Pinkel, D. (1990). Flow cytometry. Academic Press, New York.

Trouche, D., Grigoriev, M., Lenormand, J. L., Robin, P., Leibovitch, S. A., Sassone-Corsi, P., and Harel-Bellan, A. (1992). Repression of c-fos promoter by MyoD on muscle cell differentiation. Nature *364*, 79-82.

Vukicevic, S., Luyten, F. P., and Reddi, A. H. (1989). Stimulation of the expression of osteogenic and chondrogenic phenotype *in vitro* by osteogenin. Proc. Natl. Acad. Sci. USA *86*, 9793-9797.

Wang, E. A., Israel, D. I., Kelly, S., and Luxenberg, D. P. (1993). Bone morphogenetic protein-2 causes commitment and differentiation in C3H10T1/2 and 3T3 cells. Growth Factors Vol. 9: 57-71. Growth Factors *9*, 57-71.

Wang, Y., Benezra, R., and Sassoon, D. A. (1992). Id expression during mouse development: A role in morphogenesis. Dev. Dyn. *194*, 22-230.

Weintraub, H. (1994). The MyoD family and myogenesis: Redundancy networks, and thresholds. Cell *75*, 1241-1244.

Weintraub, H., Davis, R., Lockshon, D., and Lassar, A. (1990). MyoD binds cooperatively to two sites in a target enhancer sequence: occupancy of two sites is required for activation. Proc. Natl. Acad. Sci. USA *87*, 5623-5627.

Weiss, M. C., and Chaplain, M. (1971). Expression of differentiated functions in hepatoma cell hybrids: reappearance of tyrosine aminotransferase inducibility after the loss of chromosomes. Proc. Natl. Acad. Sci. USA *68*, 3026-3030.

Wewer, U. M., Albrechtsen, R., Fisher, L. W., Young, M. F., and Termine, J. D. (1988). Osteonectin/SPARC/BM-40 in human decidua and carcinoma, tissues characterized by de novo formation of basement membrane. Am. J. Patho.l *132*, 345-355.

Wolf, C., Thisse, C., Stoetzel, C., Thisse, B., Gerlinger, P., and Perrin-Schmitt, F. (1991). The M-twist gene of Mus is expressed in subsets of mesodermal cells and is closely related to the Xenopus X-twi and the Drosophila twist genes. Dev. Biol. *143*, 363-373.

Woloshin, P., Song, K., Degnin, C., Killary, A. M., Goldhamer, D., Sasso on, D., and Thayer, M. J. (1995). MSX1 inhibits MyoD Expression in Fibroblast x 10T1/2 cell hybrids. Cell 82: 611-620.

Wozney, J. M. (1992). The bone morphogenetic protein family and osteogenesis. Mol. Reprod. Dev. *32*, 160-167.

Wozney, J. M., Rosen, V., Celeste, A., Mitsock, L. M., Whitters, M. J., Kriz, R. W., Hewick, R. M., and Wang, E. A. (1988). Novel regulators of bone formation: Molecular clones and activities. Science *242*, 1528-1534.

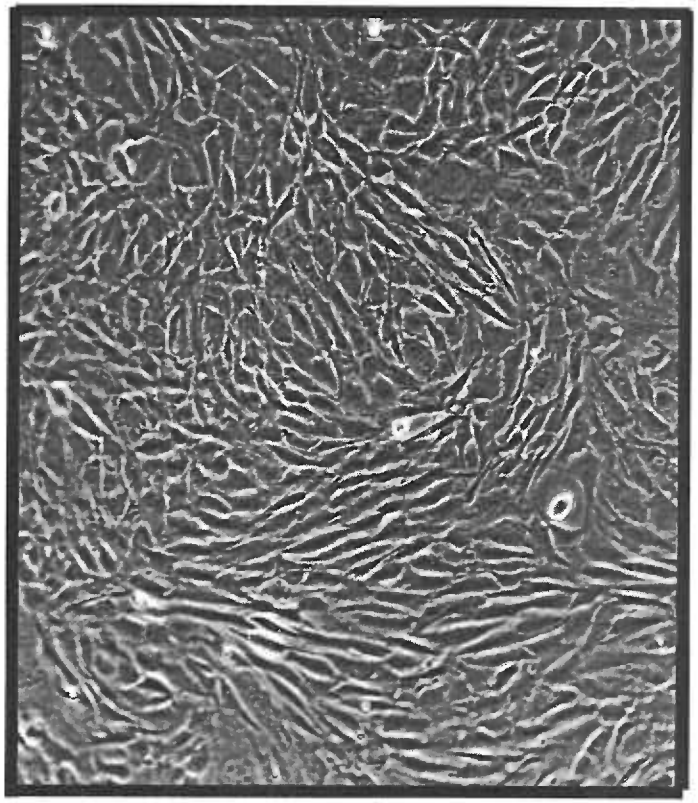
Yamaguchi, A. (1995). Regulation of differentiation pathway of skeletal mesenchymal cells in cell lines by transforming growth factor-beta superfamily. Seminars in Cell Biology. *6*, 165-173.

Yamaguchi, A., Katagiri, T., Ikeda, T., Wozney, J. M., Rosen, V., Wang, E. A., Kahn, A. J., Suda, T., and Yoshiki, S. (1991). Recombinant human bone morphogenetic protein-2 stimulates osteoblastic maturation and inhibits myogenic differentiation *in vitro*. J Cell Biol *113*, 681-687.

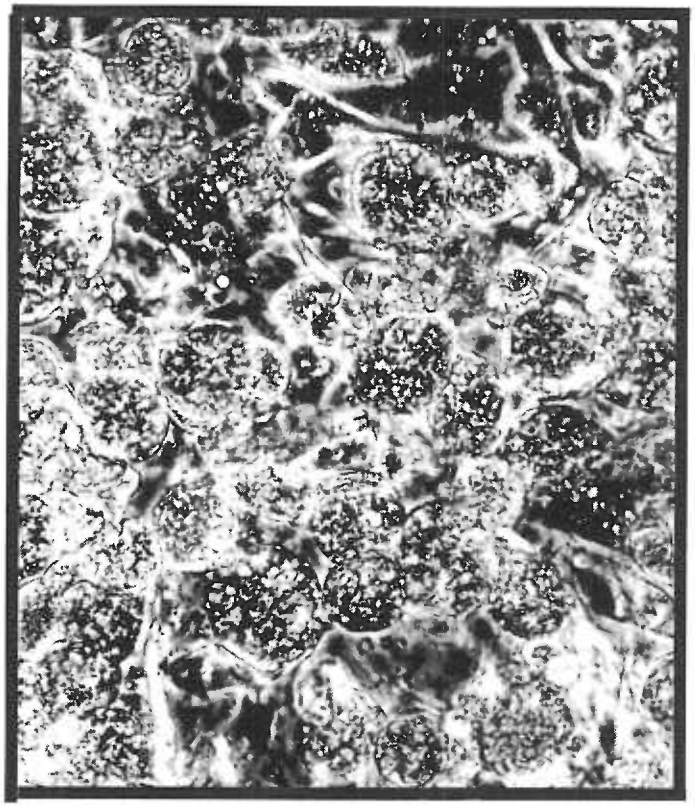
Yunis, J. J., and Chandler, M. E. (1978). High-resolution chromosome analysis in clinical medicine. Prog. Clin. Pathol. *7*, 267-288.

Zhuang, Y., Soriano, P., and Weintraub, H. (1994). The helix-loop-helix gene E2A is required for B cell formation. Cell *79*, 875-884.

Figure 1. Phase contrast photograph of fusion cell line 10HSm-15 and parental cell line C3H10T1/2 (200X). (A). C3H10T1/2 (B). 10HSm-15



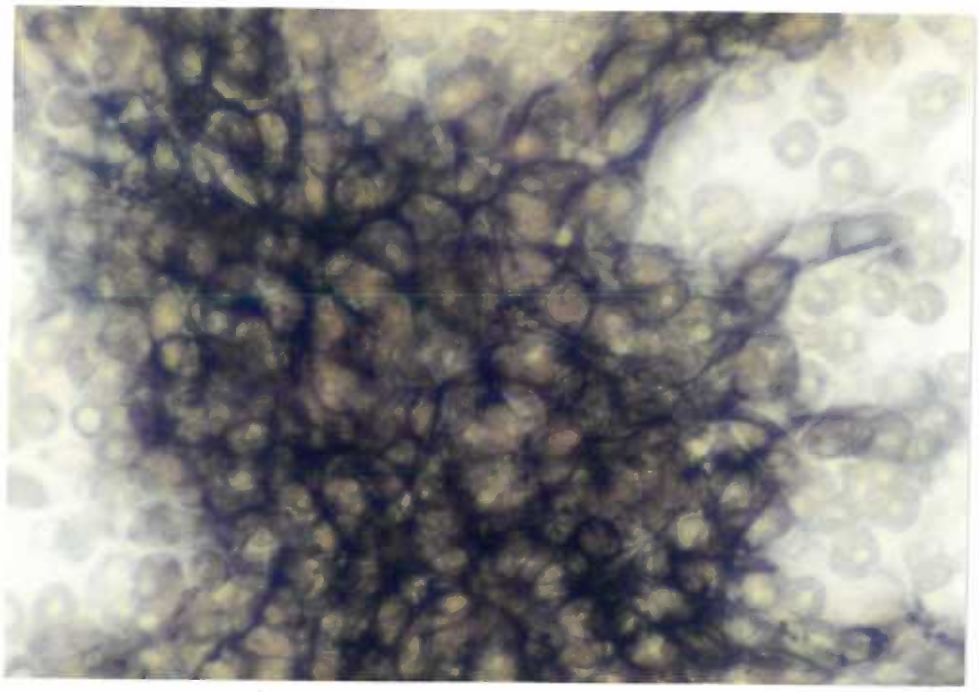
A



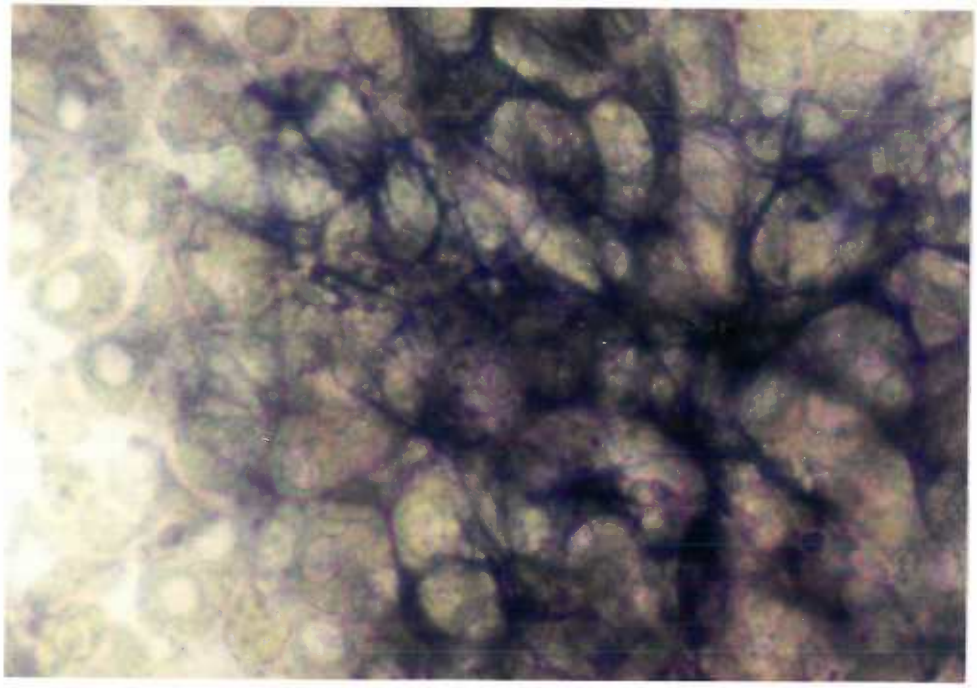
B

Figure 2. Histochemical staining of alkaline phosphtase in fusion cell line 10HSm-15 (A). 200X (B). 400X

The cells were grown in DMEM supplied with 15% calf serum. After the cells reached confluency, they were allowed to differentiate for 7 days. The cells were permeabilized in 50ug/ml lysolecithin and then stained in the NBT (Nitrobluetetrazolium) and BCIP (Bromochloroindoyl phosphate) solution.



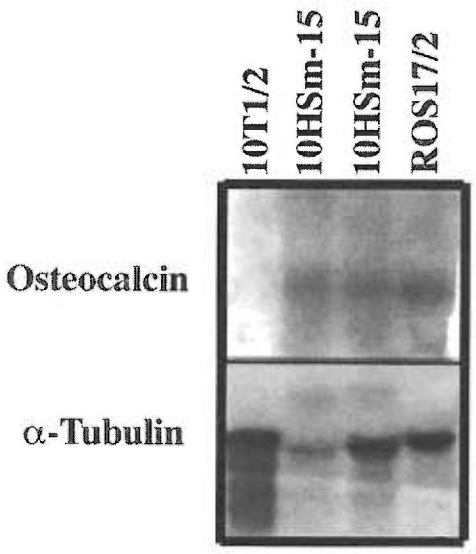
A



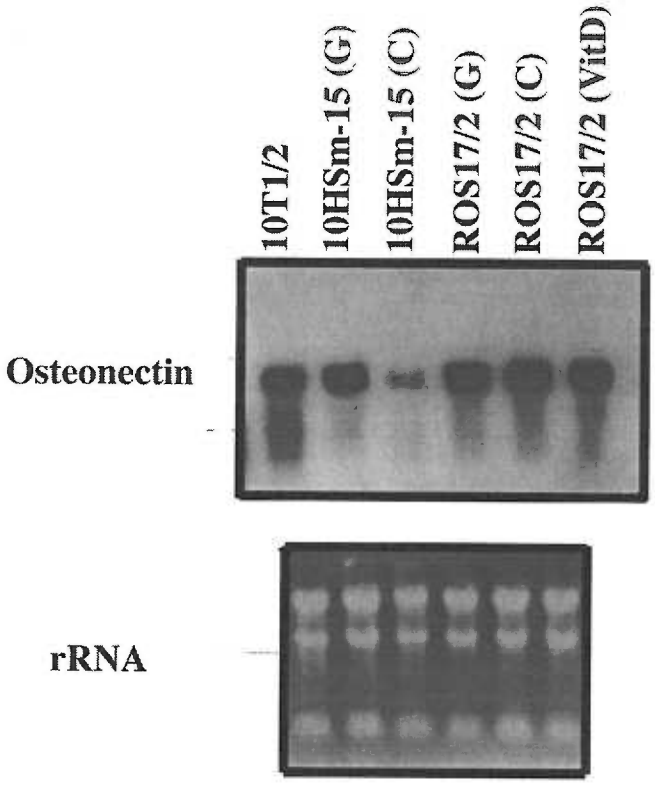
B

Figure 3. Expression of osteoblastic markers in fusion cell 10HSm-15. (A). Osteocalcin (B). Osteonectin (C). Osteopontin

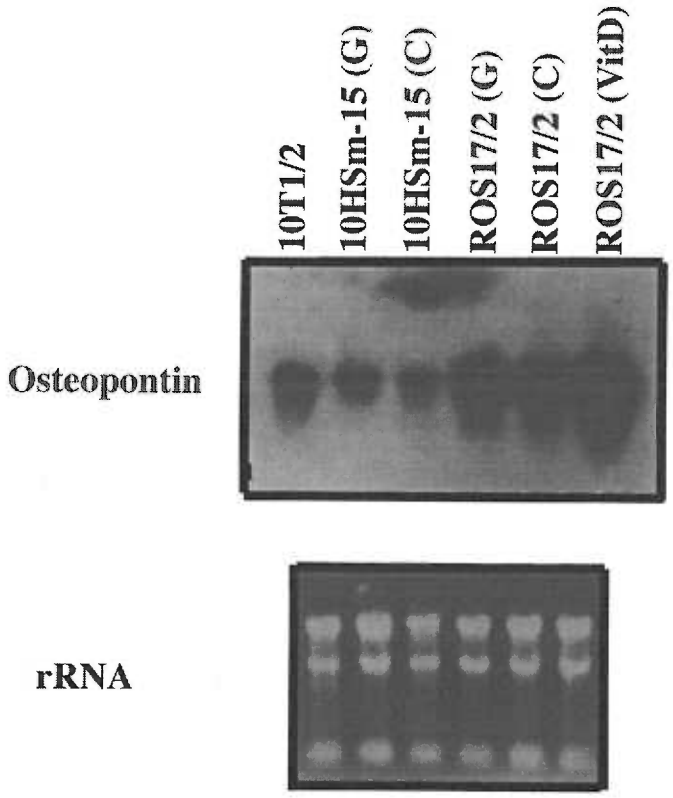
Northern Blot analysis of C3H10T1/2, 10HSm-15 and Rat osteosarcoma cell line ROS17/2. RNA was extracted from these cells and 10 ug of RNA was loaded on the gel. ^{32}P-labeled probes were used for hybridization.



A


10T1/2
10HSm-15 (G)
10HSm-15 (C)
ROS17/2 (G)
ROS17/2 (C)
ROS17/2 (VitD)
Osteonectin
rRNA

B


10T1/2
10HSm-15 (G)
10HSm-15 (C)
ROS17/2 (G)
ROS17/2 (C)
ROS17/2 (VitD)
Osteopontin
rRNA

C

Figure 4. Human chromosomes contained in the osteoblast-like hybrids

Hybrid	Human Chromosome numbers
10HSm-6	6, 8, 14, 15
10HSm-14	6, 8, 14, 15
10HSm-15	6, 8, 14, 15, 16

Figure 5. bHLH proteins expressed in the osteoblast cell line ROS17/2

bHLH Protein	Number of Times Pulled Out
Id1	1
Id2	50
Id3	4
dHAND (Th2)	4
Twist	4

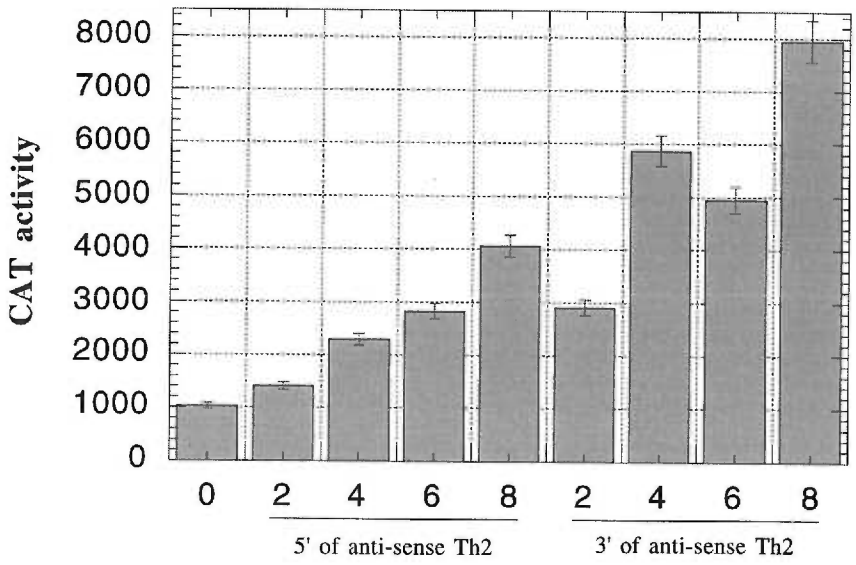
Figure 6. Expression of bHLH protein in ROS17/2. (A). dHAND (B). Twist

Northern Blot analysis of C3H10T1/2 and Rat osteosarcoma cell line ROS17/2. RNA was extracted from these cells and 10 ug of RNA was loaded on the gel. ^{32}P-labeled probes were used for hybridization.

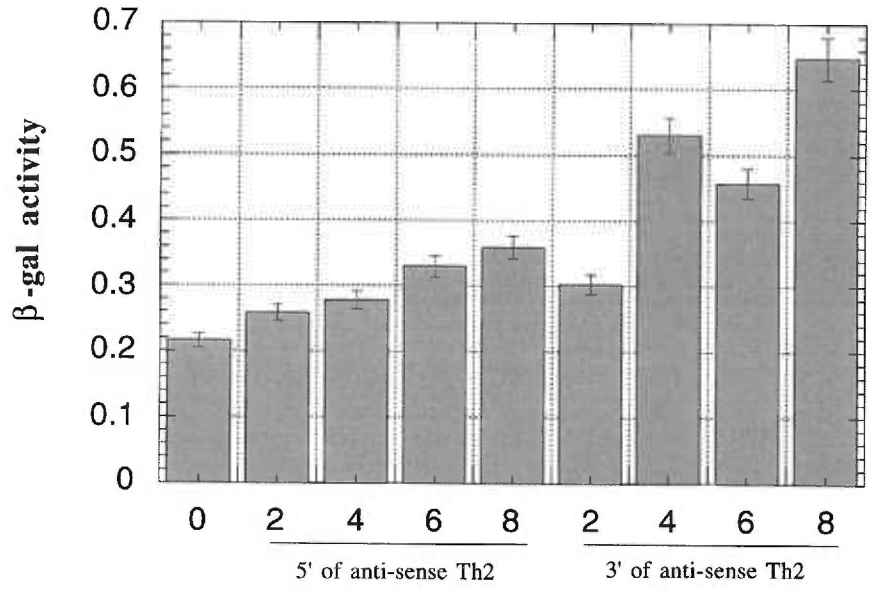
A
dHAND
α-Tubulin
10T1/2
ROS17/2 (G)
ROS17/2 (C)
B
Twist
α-Tubulin
10T1/2
ROS17/2 (G)
ROS17/2 (C)

Figure 7. Effect of dHAND (Th2) in the regulation of the osteocalcin promoter in ROS17/2. (A). Regulation of pOC-CAT by dHAND (B). Regulation of RSV-βgal by dHAND

ROS17/2 was transfected with pOC-CAT and different amount of antisense Th2 constructs. Cells were harvested after 72 hours, and CAT and βgal assays were performed. The graph is a representitive experiment, with each experiment containing duplicate plates and the error bars in the figures represent the variations between the reactions.


CAT activity
8000
7000
6000
5000
4000
3000
2000
1000
0
0 2 4 6 8 2 4 6 8
5' of anti-sense Th2
3' of anti-sense Th2
Ratio of antisense Th2:pOC-CAT

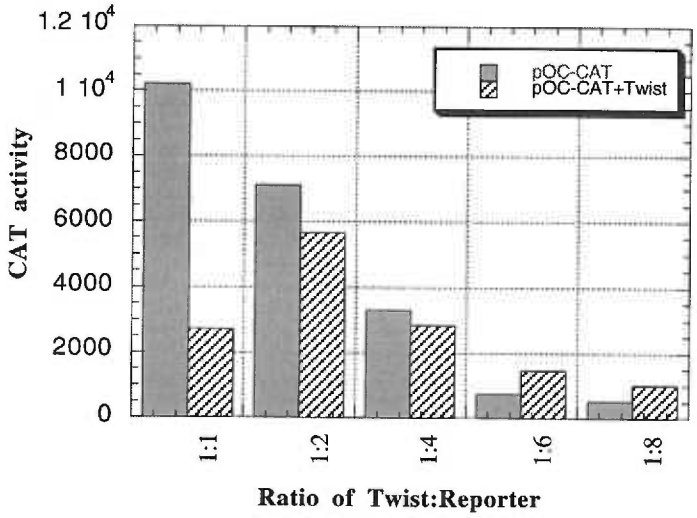
A


β-gal activity
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0 2 4 6 8 2 4 6 8
5' of anti-sense Th2
3' of anti-sense Th2
Ratio of antisense Th2:RSV-βgal

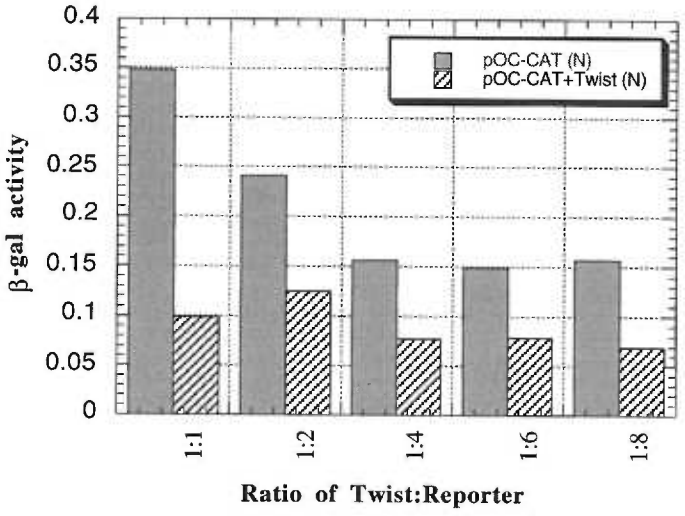
B

Figure 8. Effect of Twist in the regulation of the osteocalcin promoter in 10T1/2 cells. (A). Regulation of pOC-CAT by Twist (B). Regulation of RSV-βgal by Twist

ROS17/2 was transfected with pOC-CAT and different amount of Twist expression vector constructs. Cells were harvested after 72 hours, and CAT and βgal assays were performed. The graph is a representitive experiment, with each experiment containing duplicate plates and the error bars in the figures represent the variations between the reactions.


1.2 10^4
1 10^4
8000
6000
4000
2000
0
CAT activity
pOC-CAT
pOC-CAT+Twist
1:1
1:2
1:4
1:6
1:8
Ratio of Twist:Reporter

A


0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
β-gal activity
pOC-CAT (N)
pOC-CAT+Twist (N)
1:1
1:2
1:4
1:6
1:8
Ratio of Twist:Reporter

B

CHAPTER 2

Characterization of MDM2 Functions in the Rhabdomyosarcoma Cell Line Rh18

Abstract

Rhabodomyosarcomas are one of the most common solid tumors of childhood. Expression of the myogenic factor MyoD has been shown to be the most sensitive marker for classifying sarcomas as rhabodomyosarcomas. The rhabdomyosarcoma cell line Rh18 that has a dominant nondifferentiating phenotype. Previous studies showed that the human homologue of the mouse double minute gene #2 (MDM2) is amplified in this cell line and inhibits MyoD function.

MDM2 is amplified in approximately one third of human sarcomas. MDM2 interacts with p53 and inhibits p53-mediated transactivation. The MDM2 gene is a target of p53 transactivation. MDM2 also interacts with and inhibits pRB function. Our data show that amplification of MDM2 inhibits p53 function, and that the inhibition of MyoD is p53 independent. Amplification of MDM2 does not inhibit pRB function.

Recent studies have shown that myogenic enhancer factor 2 (MEF2) acts as a coregulator to potentiate the myogenic activities of myogenic basic helix-loop-helix (bHLH) proteins. These two classes of transcription factors synergistically activate the transcription of muscle-specific genes. The MDM2 protein binds to the MADS box of MEF2C, interfering with the synergistic activation between MEF2C and MyoD.

Introduction

1. Rhabdomyosarcoma

Rhabdomyosarcomas are childhood tumors of skeletal muscle that rarely demonstrate conclusive evidence of myogenic differentiation characteristic of mature myotubes (Johnson et al., 1965). Sarcomas have been traditionally classified as rhabdomyosarcomas on the basis of morphology and the expression of muscle-specific structural genes, such as myosin heavy chain or desmin. Expression of MyoD has been shown to be the most sensitive marker for classifying sarcomas as rhabdomyosarcomas (Dias et al., 1990) (Scrable et al., 1990). Although rhabdomyosarcomas expresses MyoD, they have a low propensity to differentiate into myotubes and the MyoD protein appears to be non-functional in inducing differentiation in these cells (Tapscott et al., 1993).

Rhabdomyosarcomas are classified into four distinct types: alveolar, pleomorphic, embryonal and botryoides (Horn and Enterline, 1958). All types of rhabdomyosarcomas have five year survival rates of approximately 70%. 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 forkhead gene family member (FKHR) in the t(2;13) translocation (Shapiro et al., 1993) (Barr 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 t(1;13)(p36;q14) in some cases of alveolar rhabdomyosarcoma (Davis et al., 1994). These fusion genes are expressed as chimeric transcripts that encode novel fusion proteins, combining functional domains usually found in separate proteins. These fusion proteins may function as transcription factors to abberrantly regulate transcription of genes controlled by PAX3 or PAX7 DNA binding sites. Both pax-3 and pax-7 genes are expressed in precursors of the skeletal musculature (Goulding et al., 1991; Jostes et al., 1991). Loss of heterozygosity on the short arm of chromosome 11, encompassing 11p15, is associated not only with embryonal

rhabdomyosarcomas (Scrable et al., 1990) but also with several other solid tumors (Newsham et al., 1991), suggesting the location of a tumor suppressor gene(s) for multiple tumor types in this region. Chromosome 12q13 abnormalities have also been observed in both alveolar and embryonic rhabdomyosarcomas (Roberts et al., 1992; Wang-Wuu et al., 1988). Utilizing Comparative Genomic Hybridization (CGH) on primary alveolar rhabdomyosarcomas, the most frequent amplicons have been localized to 2q24 and 12q13-14, with both amplifications occurring in 4 out of 10 tumors (Weber-Hall et al., 1996). The 2q24 amplicon had previously been shown to involve the MYCN gene (Dias et al., 1990a), 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) as well as in gliomas (Reifenberger et al., 1994). Mapping of these two amplicons implicates MDM2, CDK4, and SAS as likely targets of the amplification events, and amplification of these genes has consistently accompanied strong overexpression (Reifenberger et al., 1994).

2. Amplification of MDM2 inhibits muscle differentiation

One obvious phenotype of tumor cells is a lack of terminal differentiation. The rhabdomyosarcoma cell line Rh18 is an embryonic rhabdomyosarcoma cell line (Hazelton et al., 1987). Heterokaryon formation between Rh18 and 10T1/2 cells did not result in myogenesis, indicating that Rh18 cells display a dominant nondifferentiating phenotype (Tapscott et al., 1993). Transfer of a derivative chromosome 14 from Rh18 cell line into the differentiation competent myoblast cell line C2C12 inhibits muscle differentiation and the ability of MyoD to regulate its target genes (Fiddler et al., 1996). The derivative chromosome 14 contains a region of amplified DNA originating from chromosome 12q13-14, and contains several genes often amplified in sarcomas (GLI, SAS, CHOP, CDK4, MDM2). Testing the amplified genes for the ability to inhibit muscle-specific gene expression indicates that forced expression of MDM2 inhibits MyoD function, and

subsequently inhibits muscle differentiation. Expression of antisense MDM2 in C2C12 hybrids containing the derivative 14 restores MyoD dependent transcriptional activity. These results indicate that amplification of MDM2 causes a dominant non-differentiating phenotype in the rhabdomyosarcoma cell line Rh18 (Fiddler et al., 1996).

3. Murine Double Minute #2

The mdm2 oncogene was originally identified as a gene that was amplified and overexpressed in a tumorigenic derivative of mouse 3T3 cells (3T3DM cell line), with the amplified sequences located on extrachromosomal double minute particles (Cahilly-Snyder et al., 1987). The mdm2 protein contains two carboxy-terminal 'zinc-finger' motifs having significant homology to a characterized zinc-binding domain called the RING finger (Boddy et al., 1994), an acid or highly negative charged region that may have trans-activating ability and a putative nuclear localization signal. The RING-finger family of protein contain several oncoproteins including MEL18, BMI-1, RFP, PML and T18 (Freemont, 1993). Many proteins in this family are also implicated in the control of cell growth, differentiation and development.

Initial functional analysis demonstrated that overexpression of mdm2 in immortalized rodent cells is capable of tumorigenic transformation (Fakharzadeh et al., 1991). Subsequent studies revealed that overexpression of mdm2 can immortalize primary rat embryo fibroblasts and cooperate with an activated *ras* gene to transform these cells (Finlay, 1993). Amplification and overexpression of the MDM2 gene has been detected in 30% of human sarcomas (Oliner et al., 1992), indicating that this oncogene plays a role in human carcinogenesis. Further evidence supporting the conclusion that MDM2 represents a key factor in growth control pathways comes from experiments demonstrating that MDM2 protein physically associates with p53 (Barak and Oren, 1992; Momand et al., 1992; Olson et al., 1993), and inhibits p53-mediated transcriptional activation (Momand et al., 1992; Oliner et al., 1992). MDM2 also interacts with pRB and down regulates its function in cell

cycle control (Xiao et al., 1995). The relationship of these proteins with cell cycle regulation and the muscle differentiation pathway is shown in Figure 1.

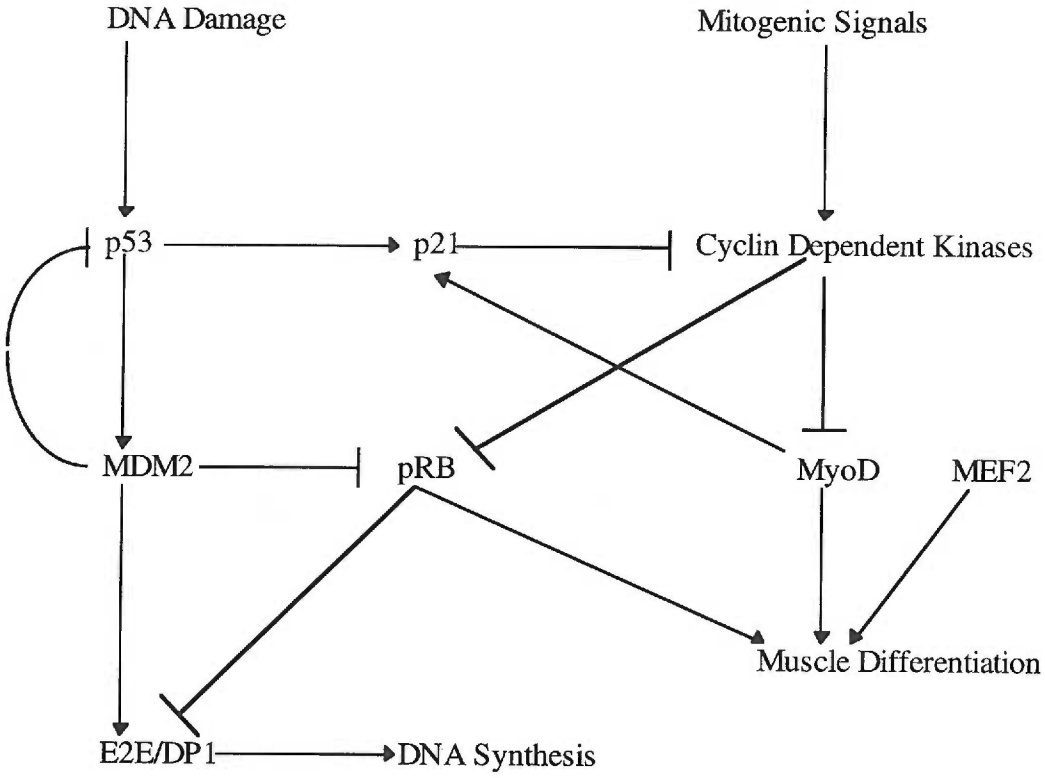


Figure 1. The relationship of cell cycle regulators with muscle differentiation

4. The interaction between p53 and MDM2

The wild-type p53 gene encodes a nuclear phosphoprotein that exhibits tumor suppressor activity (Hollstein et al., 1991; Levine et al., 1991). Structurally, p53 has been shown to be a sequence-specific DNA-binding protein (Kern et al., 1991), whose DNA-binding consensus sequence consists of two copies of a symmetric 10bp motif separated by 0-13bp (El-Deiry et al., 1992). p53 also contains an acidic activation domain near its N-terminus. p53 can inhibit oncogene-mediated transformation (Baker et al., 1990; Finlay et al., 1989).

Cell cycle checkpoints are regulatory pathways that control the order and timing of cell cycle transitions and ensure that critical events such as DNA replication and chromosomal segregation are completed with high fidelity. In addition, checkpoints respond to damage by arresting the cell cycle to provide time for repair and by inducing transcription of genes that facilitate repair. Checkpoint loss results in genomic instability and has been implicated in the evolution of normal cells into cancer cells (Elledge, 1996). DNA-damage checkpoints detect damaged DNA and generate signals that arrest cells in G1, and induces the transcription of the repair genes. Cells defective for p53 are unable to arrest in G1 in response to γ-irradiation and show reduced apotosis (Livingstone et al., 1992; Yin et al., 1992; Kuerbitz et al., 1992). Part of p53's ability to arrest in G1 results from activation of p21, a tight-binding inhibitor of the Cdks that control entry into S phase.

p53 has also been implicated as having a direct role in pathways of cellular differentiation (Shaulsky et al., 1991) and apoptosis (Clarke et al., 1993; Lowe et al., 1993; Shaw et al., 1992). Mutations in the p53 gene, with loss of tumor suppressor activity, represents one of the most common genetic events in human cancer (Hollstein et al., 1991); however, the biochemical pathways by which p53 functions in the regulation of cellular proliferation remain to be elucidated. Several groups have found that p53 protein has the properties of a transcription factor, since it can bind to specific DNA sequences (El-Deiry et al., 1992; Funk et al., 1992; Kern et al., 1991) and activates expression of genes containing p53 response elements (Funk et al., 1992; Kern et al., 1992; Zambetti et al., 1992). Similarly, p53 protein can negatively regulate the expression of several genes (Chin et al., 1992; Seto et al., 1992; Subler et al., 1992), most likely through interaction with basal transcription factors (Seto et al., 1992). Such studies indicate that p53 functions as part of the cell's transcription machinery and likely affects cellular proliferation by modulating the expression of a set of critical growth control genes (Volgelstein and Kinzler, 1992).

The MDM2 gene is a target for regulation by p53 (Barak et al., 1994; Wu et al., 1993). The first intron of the MDM2 gene contains a p53 responsive element and can be positively regulated by wild-type p53 (Barak et al., 1994; Juven et al., 1993; Wu et al., 1993). MDM2, in turn, can complex with p53 and decrease its ability to act as a positive transcription factor. In this way, the MDM2 gene is autoregulated. p53 regulates the MDM2 at the level of transcription, and MDM2 regulates p53 at the level of its activity. This creates a feedback loop that regulates both the activity of the p53 protein and the expression of the MDM2 gene. The interaction of MDM2 and p53 involves specific amino acids (Chen et al., 1993; Haines et al., 1994). The crystal structure of the amino-terminal domain of MDM2 has a deep hydrophobic cleft on which the p53 protein binds as an amphipathic α helix (Kussie et al., 1996). The interface relies on the steric complementary between the MDM2 cleft and the hydrophobic face of the p53 α helix and, in particular, on a triad of p53 amino acids-Phe^{19}, Trp^{23}, and Leu^{26}-which insert deep into the MDM2 cleft. These same p53 residues are also involved in transactivation, supporting the hypothesis that MDM2 inactivates p53 by concealing its transactivation domain.

To study the function of mdm2 during development, the mdm2 knockout mouse was generated (de Oca Luna et al., 1995; Jones et al., 1995). Mice heterozygous for the mdm2 gene deletion appeared phenotypically normal and were fertile, but mdm2 null mice are not viable and the embryonic lethality occurred around the time of implantation. However, the mdm2 and p53 double knockout mice were viable. The mdm2 null phenotype was rescued by deletion of p53. Since mdm2 is a negative regulator of p53 function (Momand et al., 1992; Oliner et al., 1993) and p53 can regulate cell growth by arresting cells in the G1 phase of the cell cycle (Baker et al., 1990), it has been proposed that the mdm2 can downregulate the ability of p53 to block progression of the cell cycle. Overexpression of exogenous mdm2 in transfected cells has been found to inhibit the ability of these cells to undergo p53-mediated growth arrest following treatment with

ionizing radiation, providing direct evidence that MDM2 is involved in inhibiting p53 function in a known pathway (Chen et al., 1996). Thus, the developmental defect in mdm2-null mice might arise as a result of failure to inhibit p53-mediated suppression of the cell cycling during the early rapid cell division period.

5. The interaction of MDM2 with pRB

Most cell types, in vivo and in vitro, can exit from the cell cycle and enter a quiescent G0 state. Following the appropriate signal, these cells can exit from this resting state and reinitiate cell proliferation. In skeletal muscle cells, cell cycle regulation plays a fundamental role in the production and maintenance of the differentiated phenotype. Proliferating myoblasts do not express the differentiated phenotype. In response to differentiation signals, myoblasts permanently withdraw from the cell cycle and fuse into multinucleated myotubes, concomitant with the expression of muscle-specific gene products. This process is controlled by regulatory interactions involving MyoD family members and various proteins (Yun and Wold, 1996). 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. Furthermore, MyoD transactivation of muscle-specific genes to high levels requires pRB (Gu et al., 1993; Novitch et al., 1996) or the pRB related protein p107 (Schneider et al., 1994). pRB-deficient muscle cells can activate normal levels of "early" differentiation markers (i.e., myogenin and p21); however, expression of "late" differentiation markers (i.e., myosin heavy chain) is attenuated in these cells. It has been shown that MyoD induces growth inhibition within the G_1 phase of the cell cycle (Weintraub et al., 1989). This MyoD-mediated cell cycle arrest is mediated by its interaction with pRB, a cell cycle regulator (Gu et al., 1993; Martelli et al., 1994).

pRB is a nuclear phosphoprotein whose phosphorylation is regulated during the cell cycle and cell differentiation (Weinberg, 1995). Cells in G_0 and G_1 contain an unphosphorylated or underphosphorylated form of RB, while specific phosphorylation of

pRB occurs at the G_1/S boundary and is associated with cell proliferation. The growth-suppressing effects of pRB are exerted in the G_0/G_1 phase of the cell cycle and is inactivated by phosphorylation. The RB family contains pRB and its two relatives, p107 and p130. They work together to regulate the activity of the transcription factor E2F (Muller, 1995). E2F, with its dimerization partner DP1, has a critical role in cell growth control. The pRB protein efficiently interacts with the E2F1/DP1 dimer. This ternary complex binds to E2F sites and acts as a transcription repressor to prevent the transactivation of E2F target genes. Such complexes are found preferentially in G_0 and G_1. The release of pRB binding to this dimer converts them into transcriptional activators and consequently activates the transcription of genes required for entry into S phase. Therefore, pRB is an important regulator of the cell cycle and of the ability of cells to enter and remain in the G_0 state.

During skeletal muscle differentiation, pRB is involved in the myogenic function of MyoD (Gu et al., 1993). Recent studies showed the modulation of pRB mRNA levels during myogenic differentiation of the murine myoblast C2C12 cell line and provide evidence that MyoD enhances pRB gene transcription (Endo and Goto, 1992) (Martelli et al., 1994). In differentiated cells, unphosphorylated pRB and MyoD bind to each other specifically and with high affinity in vitro and in vivo (Gu et al., 1993). The sequences required for MyoD and pRB binding include a portion of the pRB pocket and bHLH domains of MyoD, respectively, which are also known to be involved in other protein-protein interactions important for the biological functions of these molecules. This interaction is crucial for the induction of both differentiation and permanent withdrawal of muscle cells form the cell cycle. Unlike their wild-type counterparts, multinucleated myotubes deficient in pRB (pRB-/-) can still reenter the cell cycle upon growth factor stimulation (Novitch et al., 1996; Schneider et al., 1994). In addition, an inhibitor protein with a helix-loop-helix domain, Id-2, also interacts with pRB. This interaction has the

opposite effect on the cell cycle in that it enhances cell proliferation (Iavarone et al., 1994). Therefore, pRB plays an important role in the regulation of muscle differentiation.

Recent studies have shown that MDM2 interacts with pRB both physically and functionally to inhibit pRB growth regulatory function (Xiao et al., 1995). MDM2 interacts with the two cooperating transcription factors, E2F1 and DP1, which are involved in S-phase progression (Martin et al., 1995). These results indicate that MDM2 regulates cell cycle progression through a set of cell cycle regulators.

6. Myogenic coregulators: myocyte enhancer factor 2

Regulation of skeletal muscle determination and differentiation in vertebrates centers on a core regulatory network which is composed of two families of transcription factors, the MyoD group basic helix-loop-helix (bHLH) muscle regulatory factors (MRFs) and the myocyte enhancer factor 2 (MEF2) group of MADS-box regulators. Members of this network interact with each other genetically and physically, and together they cooperate to positively regulate transcription of downstream muscle-specific genes.

The MRF family contains four members: MyoD, myogenin, Myf-5 and MRF4. The MRF genes encode nuclear phosphoproteins that contain a conserved central protein motif referred to as the basic helix-loop-helix domain (Edmondson and Olson, 1993). The α-amphipathic helices of the bHLH motif, separated by a variable loop region, are responsible for protein dimerization, while the basic region provides the contact points with an appropriate DNA target. For the bHLH MRF proteins, the consensus DNA sequence -CANNTG- referred to as an E-box, serves as the molecular target, although additional flanking nucleotides also have a role in DNA target specificity (Blackwell and Weintraub, 1990). Binding of the MRFs to E-box elements located within the regulatory regions of most muscle-specific genes is often sufficient to activate muscle gene expression. The MRFs function as heterodimers forming a complex with a second bHLH

protein from the E-protein family, such as E12, E47, E2-5, or HEB (Shirakata et al., 1993). These E-proteins are expressed in a wide variety of cell types.

Different MRF genes serve at least two distinct developmental functions (Megeney and Rudnicki, 1995). Myf-5 and MyoD jointly act as determination genes whereas myogenin functions as a differentiation gene. Thus, myf5 and MyoD double knockouts fail to either produce or sustain a significant population of myoblasts. Myogenin-null mice are highly (though not entirely) deficient in muscle differentiation, although they have a large pool of myoblast precursor cells where muscle fibers would normally be. Expression of MyoD or Myf-5 is required to generate the myoblast population, while myogenin is required to initiate terminal differentiation events associated with muscle development. The function importance of MRF4 is not clear yet.

A second class of muscle transcription factors that control myogenic events is the MEF2 family, which belongs to the MADS superfamily of DNA binding proteins named for the first four factors in which this domain was identified: MCM1, which regulates mating type-specific genes in yeast, Agamous and Dificiens, which act as homeotic factors that control flower development, and Serum response factor, which controls serum-inducible and muscle-specific gene expression (Pollock and Treisman, 1991; Shore and Sharrocks, 1995). MEF2 was identified originally in C2 myotube cultures by its ability to bind to a conserved A/T-rich DNA sequence, -CTA[A/T]$_4$TAG/A- (MEF2 site), that is present in the regulatory regions of many muscle-specific genes (Gossett et al., 1989). The mammalian MEF2 family is comprised of four distinct genes, named MEF2A, MEF2B, MEF2C, and MEF2D (Olson et al., 1995). Members of the MADS superfamily share a common 56-amino acid motif, referred to as the MADS box, that is responsible for DNA binding and dimerization. In addition, MEF2 factors contain an unique conserved 29-amino acid sequence called the MEF2 domain, which also may dictate DNA target specificity. The MEF2 proteins interact with other members of the MEF2 family to transcriptionally activate numerous muscle-specific genes. Unlike the skeletal muscle-specific expression exhibited

by the MRFs, the MEF2 genes are expressed in a broader range of cell types, including brain and neural crest cell derivatives as well as skeletal, cardiac, and visceral muscle (Edmondson et al., 1994). After birth, MEF2A, MEF2B, and MEF2D transcripts are expressed ubiquitously, whereas MEF2C transcripts are restricted to skeletal muscle, brain, and spleen (Martin et al., 1993; McDermott et al., 1993).

Recent studies have provided evidence for a complex regulatory network between members of the muscle bHLH and MEF2 protein families. MEF2 DNA binding sites, for example, often are positioned within close proximity to MRF binding sites (E-boxes) in the regulatory regions of many muscle genes. Forced expression of myogenin or MyoD in 10T1/2 fibroblasts induces MEF2 DNA binding activity, suggesting that the MEF2 genes are regulated by the MRFs in a linear, regulatory pathway (Cserjesi and Olson, 1991). Reciprocally, MEF2 proteins are capable of transcriptionally activating expression of the myogenin and MRF4, raising the possibility that members of the MEF2 family regulate MRF genes expression, and vice versa (Cheng et al., 1993) (Naidus et al., 1995). When MEF2 and MRF proteins are coexpressed in cells, they cooperate to *trans*-activate expression of reporter genes containing both MEF2 and E-box elements in a synergistic fashion (Kaushal et al., 1994; Li and Capetanaki, 1991; Naidus et al., 1995). Direct evidence that the cooperativity between MEF2 proteins and myogenic bHLH proteins regulates muscle gene expression was demonstrated recently (Molkentin et al., 1995). This synergy is mediated by direct protein-protein interactions between MEF2 factors and heterodimers formed between myogenic bHLH factors and E proteins (Molkentin et al., 1995). This interaction was mapped to the amino-terminal 105 amino acids of MEF2C, which encompasses the MADS-box and the MEF2 domain, and to the bHLH domain of myogenin. This interaction permitted either factor to activate transcription through the binding site of the other. Another MADS-box transcription factor, serum response factor (SRF), has also been shown to interact physically with myogenin/E12 heterodimers (Groisman et al., 1996). This interaction was also reported to be mediated by the DNA

binding domains of these two classes of transcription factors. Together, these findings demonstrate that the myogenic bHLH proteins interact with MADS-box proteins to regulate muscle gene expression and suggests a mechanism whereby interaction between these two heterologous factors specify promoter-enhancer communication to recruit more efficiently the basal transcription machinery.

Materials and Methods

1. Cell lines: C2C12 cells are murine myoblast cell line and were from the American Type Culture Collection. Rh18 and Rh30 cells are rhabdomyosarcoma cell lines and were provided by P. Houghton (Hazelton et al., 1987). C2(Rh1811) cells are secondary microcell hybrids between C2C12 and Rh18 cells (Fiddler et al., 1996). They contain the derivative chromosome 14 which contains a region of amplified DNA originating from chromosome 12. C2(Rh30) is the hybrids between C2C12 and Rh30 cells (Fiddler et al., 1996). All cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% calf serum (Hyclone Laboratories). The cells were differentiated in DMEM containing 2% horse serum.

2. Myosin heavy chain immunostaining and BrdU labeling: After differentiation, cells were stimulated with DMEM containing 15% fetal bovine serum (FBS) and 10 μM BrdU (5-Bromodeoxyuridinine) (Boehringer Mannheim Corp.) for 24-48 hours. Cells were washed three times with PBS, fixed for 1 minute in ice-cold AFA (70% ethanol, 3.7% formaldehyde, 5% glacial acetic acid), and then washed three times with PBS. Air dry the plate, add 0.07N NaOH for 2 minutes and then neutralize the base with PBS (pH8.5). The cells were preincubated with PBS/1% horse serum for 30 minutes. Myosin heavy chain (MHC) was detected using a 1:10 dilution of the anti-MHC antibody MF-20 and 1:200 dilution of rhodamine-conjugated rabbit anti-mouse IgG antibodies (Sigma). After the secondary antibody incubation step, the cells were put into PBS/0.5% Tween 20 containing 1:10 dilution of FITC-conjugated monoclonal Anti-BrdU (Becton Dickinson) for 30 minutes. Wash the cells with PBS and then stain the nuclei with 4′, 6′-diamidino-2-phenylindole (DAPI) (1 mg/ml) for 1-2 minutes. Mount the plate with anti-fade (2.3% 1,4-Diazabicyclo[2,2,2] octane (Sigma), 90% glycerol, 0.02 M Tris-Cl

(pH8.0)) and coverslide. Cells were visualized under UV fluorescence with DAPI, FITC and rhodamine filters (Zeiss).

3. Cells, DNA damage and cell cycle analysis: cells were irradiated in a ^{137}Cs γ-irradiator at 100rads/min (1rad=0.01 Gy) for total 20 Gy and refed with fresh medium. After 17 hours' incubation, the cells were trypsinized from the plates and washed twice with PBS/1% BSA. Resuspend the cells in 200μl PBS on ice and slowly add cells into 5 ml 70% ethanol (-20 °C) while maintaining a vortex. Incubate the cells on ice for 30 minutes, resuspend them in 2N HCl/0.5% Triton X-100 for 30 minutes at room temperature, and then resuspend the cells in 0.1 M $Na_2B_4O_7.10H_2O$ (pH8.5) to neutralize the acid. The cells were then stained with 5 μg/ml of propidium iodide (Sigma). Analyze the cells on a FACS brand flow cytometer (Becton Dickinson Immunocytometry Systems) at laser excitation of 488 nm.

4. In vitro transcription and translation: the MEF2C gene and its mutants, MyoD, p53 and SRF were cloned downstream of the T7 polymerase promoter. MyoD and MyoD-VP16 were cloned downstream of the T3 polymerase promoter. The deletion mutants except 1-95 and site-mutants of MEF2C were provided by Dr. Eric Olson. All the proteins were transcripted and translated by using the TNT® coupled reticulocyte lysate system (Promega) and radiolabelled with ^{35}S-methionine. An aliquot of the products was used to run on a standard 8-10% SDS-polyacrylamide gel and the protein products can be visualized by autoradiography.

5. MBP (maltose binding protein)-binding assay: inoculate 400 ml BL21:UBS520 (provided by Dr. Stan Hollenberg) containing the pMAL-MDM2 plasmid

(*mdm2* gene is fused in frame with the Maltose binding protein MALE signal sequence) in bacterial LB medium supplemented with Kanomysin and Ampicillin for selection (1:100 dilution from overnight small culture). When the OD_{600} of the culture is about 0.6, induce the cells with 0.5 mM IPTG (isopropylthio-β-galactoside) for 3 hours. Harvest the cells and resuspend them in 5 ml PBST (PBS plus 0.1% Triton X-100) supplemented with 1 mM EDTA, 1 mM PMSF, and 14 mM β-Mercaptoethanol (PBST/supplements). Sonicate the cells and collect the supernant. Purify the MBP-MDM2 fusion protein with amylose resin (New England Biolabs). Briefly, prewash the resin with the PBST/supplements for four times and then add the resin to the supernant (4 ml resin to 5 ml supernant). Rock the mixture at 4 °C for 1 hour, wash the resin three times with PBST/supplements and then three time with NETT (20 mM Tris-Cl [pH8.0], 100 mM NaCl, 1 mM EDTA, 0.2% Triton X-100). Take an aliquot of the resin and elute with 10 mM maltose to check the products on the 8-10% SDS-polyacrylamide gel. To do the MBP-binding assay, add about 20 μg protein coated amylose resin, 5 μl in vitro translated protein and 45 μl NETT binding buffer to a total volume of 55 μl. Incubate the mixture at 4 °C for 2 hours with rocking and then wash three times with NETT. Remove the final wash as much as possible. Resuspend the pellet in 10 μl 1xSDS protein loading buffer (50 mM Tris-Cl [pH6.8], 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and heat it at 95 °C for 2 minutes. Analyze the supernant on 8-10% SDS-polyacrylamide gel. Entensify the gel with Entisifier A and B (NEN) and then dry it. Expose the gel on film (Kodak) for overnight.

6. **Gel electrophoresis mobility assay:** the double-stranded oligonucleotide containing four tandem copies of the MEF2 DNA binding site: 5′-PTAAAAATAA C TAAAAATAACTAAAAATAACTAAAAATAAC-3′. To prepare the radiolabelled the

probe, 25 ng of the sense strand was incubated with 20 μCi of ^{32}P-labeled γATP and 5U Polynucleotide Kinase (Boehringer Mannheim) at 37 °C for 1.5 hours and then the reaction was incubated at 80 °C for 10 minutes. Cooling the reaction on ice and add 50 ng of the antisense strand of the oligonucleotide and NaCl to a final concentration of 0.1 M. The reaction was incubated at 95 °C for 5 minutes, room temperature for 5 minutes and on ice for 5 minutes. Add 30 μl of TE into the reaction and spin it through a Sephadex G-50 column to purify the probe. To do the gel shift assay, the in vitro translated MEF2C protein was incubated with the MBP-MDM2 protein and NETT buffer in a total volume of 40 μl for 2 hours. After the incubation, the mixture was added 8 μl of 5XGel shift reaction buffer (0.1 mM Hepes [pH7.6], 0.5 mM KCl, 5 mM EDTA, 15 mM $MgCl_2$, 5 mM DTT, 50% glycerol, 2.5% NP40), 2.5 μg poly(dI-dC) and $1X10^5$ cpm of the radiolabelled oligonucleotide. The reaction was incubated at room temperature for 15 minutes and then was added 1 μl of 0.05% Bromphenol Blue (Sigma). One half of the reaction was loaded onto 2% low melting agarose gel (Boehringer Mannheim) in the TBE running buffer (50 mM Tris Base, 50 mM Boric acid, 1 mM EDTA). The gel was dried and expose to a X-ray film (Kodak).

Results

1. Amplified MDM2 inhibits p53 dependent functions in muscle cells

Mutation of p53 is the most common genetic event in the development of human neoplasia, occurring in approximately 60% 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 transactivation 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 tested whether the DNA damage induced p53 pathway was altered in the derivative 14 hybrids containing the amplified MDM2 gene.

In response to DNA damage, mammalian cells arrest the cell cycle. Although the mechanisms by which cells arrest at different points in the cell cycle have not been fully elucidated, it is clear that G1 arrest is dependent upon the p53 gene product. To examine the effects of amplified MDM2 on cell cycle arrest, we assayed the ability of the parental C2C12 cells and the derivative 14 hybrids to arrest the cell cycle following γ-irradiation by flow cytometry. Figure 2 shows that the parental C2C12 cells retain a prominent G1 arrest (2N) and do not accumulate tetraploid (8N) cells following irradiation. In contrast, the hybrid C2(R1811)-1, which contains amplified MDM2, shows a dramatic decrease in the G1 population and contains a significant number of tetraploid cells following irradiation, while the p53 independent G_2 checkpoint is still maintained. These results are consistent with previous observations showing that forced expression of MDM2 inhibits G1 arrest and apoptosis functions of p53 (Chen et al., 1996). Since G1 arrest following DNA damage is p53 dependent, we conclude that the parental C2C12 cells have functional p53, while the hybrids retaining amplified MDM2 do not. These results are consistent with the

known functions of MDM2 and suggest that MDM2 inhibits muscle differentiation in the derivative 14 hybrids by inhibiting p53.

2. MDM2 amplified derivative 14 hybrids maintain wild type pRB cell cycle arrest function in myotubes

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 (Olson, 1992). During muscle differentiation, pRB expression increases (Endo and Goto, 1992) (Martelli et al., 1994) and assumes a hypophosphorylated, activated state (Gu et al., 1993). pRB plays an important role in the production and maintenance of the terminally differentiated phenotype of muscle cells. Inactivation of pRB in terminally differentiated cells allows them to reenter the cell cycle. In addition to its involvement in the myogenic activities of MyoD, pRB is also required for the cell growth-inhibitory activity of this myogenic factor. Furthermore, activation 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). Because MDM2 interacts with pRB and inhibits its growth regulatory functions (Xiao et al., 1995), it is likely that MDM2 may block pRB-related functions in the derivative 14 hybrids.

To determine whether MDM2 amplification affects the role of pRB in the maintenance of the terminally differentiated phenotype of myotubes, we utilized the BrdU incorporation assay to analyze DNA synthesis following differentiation. Wild type C2C12, derivative chromosome 14 clone C2(R1811)-1, and the CDK4 amplified clone C2(Rh30)-3 cells were cultured in differentiation medium for 48 hours and refed with medium containing high serum (15% FBS) plus 10 μM BrdU. The cells were incubated for 24 hours and then fixed for evaluation of BrdU uptake and MHC expression (Figure 3). Compared to the wild type C2C12 cells, the derivative 14 hybrid differentiated poorly.

Unlike their wild type counterparts, these cells did not fuse to form multinucleated myotubes despite their MHC expression. These MHC-expressing cells immunostained negatively with the BrdU antibody after restimulation with serum growth factor-rich medium. This is similar to their wild type counterparts. We also quantitated BrdU incorporation into proliferating undifferentiated cells and differentiated cells after 24 and 48 hours restimulation with growth medium both in the wild type and the hybrid cells (Figure 4). While very little BrdU incorporation was detected in the differentiated cells of all three cell lines, over 60% of the non-differentiating cells from each cell line reentered the cell cycle and incorporated BrdU after 48 hours. This result indicates that in the MDM2 overexpressing cells, pRB is still functioning to maintain cell cycle arrest and prevents the cells from entering S phase. This result also suggests that MDM2 does not affect the ability of pRB to maintain the terminally differentiated phenotype of muscle cells.

3. MDM2 interacts with MEF2C in vitro

Muscle-specific transcription by MyoD has been shown to require a second factor (a "recognition factor") that recognizes the basic region bound to DNA (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 bHLH domain of MyoD and facilitate transcriptional activation through a MyoD 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, thus MEF2C contains all of the properties of the "recognition factor". Previous results (Fiddler, T. unpublished data) showed that inhibition by MDM2 involves the MyoD DNA binding domain and the MyoD DNA binding site and that this inhibition does not involve loss of MyoD protein. Equivalent amounts of MyoD DNA binding activity was also found in the derivative 14 hybrids by using gel mobility shift assays combined with antibody "supershift".

Furthermore forced expression of MEF2C restored MyoD activity in the presence of high levels of MDM2. The above results suggest that MDM2 is inhibiting MyoD activity indirectly, and that MEF2C is a likely target of MDM2 action.

To determine whether MDM2 interacts with MEF2C, we fused full length MDM2 to the E.coli maltose binding protein (MBP) and tested for interaction with in vitro translated MEF2C in an MBP pull down assay. Figure 5 shows that MBP-MDM2 interacts with MEF2C, while MBP alone does not. In addition, in vitro translated p53 and MyoD were also included in this assay as controls. Consistent with the known interaction between MDM2 and p53, MBP-MDM2 pulls down p53. We have not detected an interaction between MDM2 and MyoD in the MBP pull down assay (Figure 5), nor have we detected an MDM2-MyoD interaction with co-immunoprecipitation or yeast two hybrid assays (data not shown). These data indicate that MDM2 interacts with MEF2C, but not with MyoD in vitro.

MDM2 interacts with the activation domains of both p53 (Oliner et al., 1993) and E2F1 (Martin et al., 1995). To begin to determine the region of MEF2C required for MDM2 interaction, we tested various MEF2C deletion mutants for interaction with MDM2 (Figure 6B). Figure 6A summarizes the results of these assays. We show here that unlike p53 and E2F1, the deletion of the activation domain of MEF2C (1-117) does not abolish the ability of MEF2C to interact with MDM2. In contrast, deletion of the MADS box and the MEF2 domain (Δ1-95) results in complete loss of the MDM2 interaction, whereas deletion of the MADS box residues 40-57 or deletion of the MEF2 domain (Δ58-85) alone had little or no effect on the MDM2 interaction. These data indicate that the N-terminus of MEF2C is the target for interaction with MDM2, and also indicate that the MADS box and not the activation domain of MEF2C is responsible for this interaction. We also detected an interaction between MDM2 and another MADS box protein, SRF in our MBP-binding

assay (Figure 5). This may indicate that MDM2 can bind to all MADS-box protein family members and interrupt their function.

Previous studies showed that several amino acids at the N-terminus are critical for MEF2C DNA-binding and dimerization function. Mutation of amino acid Lys-5 of MEF2C resulted in a complete loss of DNA binding activity without a loss in dimerization potential and mutation of Lys-4, Arg-15, Arg-17 and Lys-23 resulted in a partial loss of DNA binding activity without a loss in dimerization potential (Molkentin et al., 1996). In order to determine whether single amino acid mutations can disrupt the MDM2-MEF2C interaction, we tested these mutants for interaction with MDM2 (Figure 7). The results showed that all of these mutants still bind to MDM2 in the pull down assay. This indicates that the interaction interface between these two proteins may involve a region of the MEF2C MADS box not mutated in this limited set of mutants.

4. MDM2 does not block MEF2C DNA binding ability

Our study showed that MDM2 interacts with the MADS box of MEF2C and specifically the N-terminus of the MADS box. This region of the MADS box is critical for both MEF2C DNA binding site and dimerizing with other cofactors (Molkentin et al., 1996). Mutations of this region either lose DNA binding or dimerization or both. We used gel electrophoresis mobility shift assay to test whether binding to MDM2 can abolish the DNA binding ability of MEF2C. Figure 8 shows that MEF2C is still able to interact with its DNA binding sequence in the presence of MDM2. MDM2 does not interfere with the DNA binding ability of MEF2C. We speculate that MDM2 may block the ability of MEF2C to cooperate with myogenic factors to activate muscle differentiation pathway.

Discussion and Conclusion

Cell proliferation and differentiation are mutually exclusive events in a variety of cell types. In muscle cells, the decision to divide or differentiate is determined by a balance of opposing cellular signals. The skeletal muscle malignancy rhabdomyosarcoma provides an example of the antagonism between signals for growth and differentiation within the myogenic lineage. Rhabdomyosarcoma cells express the muscle differentiation factors MyoD and myogenin to varying degrees, but they show only limited expression of genes associated with terminal differentiation (Dias et al., 1990). The phenotype of these cells suggests that they are derived from myogenic progenitors committed to a myogenic fate but are arrested at an early step in the differentiation pathway due to altered growth control. In our laboratory, we have been studying the rhabdomyosarcoma cell line Rh18 and have identified a genetic locus that is capable of inhibiting muscle differentiation (Fiddler et al., 1996). This study showed that MDM2 is amplified in this cell line and is responsible for the nondifferentiating phenotype.

The oncogenic properties of MDM2 have been postulated to result from direct interaction with several cell cycle regulatory proteins. MDM2 interacts 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). In addition, MDM2 interacts with the activation domain of E2F1 resulting in stimulation of E2F1/DP1 transcriptional activity (Martin et al., 1995). Furthermore, MDM2 has been shown to interact with pRB, resulting in stimulation of E2F1/DP1 transcriptional activity and inhibition of pRB growth regulatory function (Xiao 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 E2F1/DP1. Thus, the most obvious role for amplification of MDM2 in tumorigenesis would be to inactivate p53 and /or pRB. We initiated a series of

experiments to test whether amplification of MDM2 in our derivative 14 hybrids would affect the function of these two proteins.

1. Amplified MDM2 inhibits p53 dependent functions in rhabdomyosarcoma cells, but p53 is not required for inhibition of MyoD activity

Since p53 is involved in the G_1 checkpoints (Livingstone et al., 1992) (Yin et al., 1992) (Kuerbitz et al., 1992) and DNA damage or unfavorable conditions can induce p53 protein expression which in turn induces p21 expression. The hybrids that contain amplified MDM2 have lost the p53 dependent G_1 arrest after γ-irradiation, while they still maintained the p53 independent G_2 arrest. These results are consistent with previous work that showed overexpression of MDM2 inhibits the G_1 arrest and apotosis functions of p53 (Chen et al., 1996). However, MDM2 does not function through p53 to repress MyoD activity. MyoD can still transactive its target sites in the absence of p53, and MDM2 is still able to repress the MyoD function without p53 (Degnin unpublished data). We also observed that forced expression of p53 in the derivative 14 hybrids does not restore MyoD activity (Fiddler unpublished result). 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). Expression of MyoD can activate the Cdk inhibitor p21 independent of p53 and correlated with cell cycle withdrawal. Therefore, p21 expression is involved in two types of regulatory pathways: p21 expression during development is p53-independent and regulated by other transcriptional factors for normal developmental processes; and, p53 appears to be necessary for p21 induction following DNA damage, a function that may be critical for its tumor suppressor properties.

The p53 tumor suppressor helps maintain the genomic integrity of the cell as it coordinates the cellular response to DNA damage by inducing cell cycle arrest or apoptosis. Accordingly, inactivation of p53 is one of the most common events in neoplastic transformation. In about half of all cancer cases, p53 is inactivated by mutations and other

genomic alterations (Greenblatt et al., 1994), and in many of the remaining cases it is functionally inactivated by the binding of the cellular MDM2 oncoprotein. Because the p53 gene is the most frequently mutated gene in human cancer and cells must undergo numerous genetic changes to generate a solid metastatic tumors, MDM2 may repress the p53 dependent cell cycle arrest function and thereby increase the mutation rate. It is also well-known that loss of p53 predisposes cells to drug-induced gene amplification and decreases the fidelity of mitotic chromosome transmission (Livingstone et al., 1992; Yin et al., 1992). Duplication of the centrosome normally begins at the G_1-S boundary, but in the absence of p53, multiple centrosomes appear to be generated in a single cell cycle, ultimately resulting in aberrant chromosomal segregation during mitosis (Fukasawa et al., 1996). Amplification of 12q13 is thought to be due to some growth advantages. Overexpression of MDM2 blocks p53 transactivation function and also inhibits the differentiation pathway of myoblasts by interacting with other factors, such as MEF2C. In conclusion, overexpression of MDM2 in the derivative 14 hybrids blocks p53 function in the cell cycle checkpoint, but loss of muscle differentiation is p53 independent.

2. Amplification of MDM2 does not inhibit pRB dependent terminal differentiation

The retinoblastoma susceptibility gene product pRB is generally believed to be an important regulator in the control of cellular proliferation and regulation of the cell cycle (Weinberg, 1995). Deletion or mutational inactivation of pRB is correlated with the genesis of a variety of human cancers including retinoblastoma, osteosarcoma, and carcinomas of the breast, bladder, and lung. The pRB protein may also be sequestered by viral oncoproteins, such as simian virus 40 larger tumor antigen, via conserved regions of the oncoproteins that are essential for viral-induced transformation (Decaprio et al., 1989; Dyson et al., 1989).

Change in pRB phosphorylation, with accumulation of the growth-suppressing unphosphorylated form of pRB, is one of the early events leading to muscle cell differentiation. Most of the pRB protein is phosphorylated in cycling myoblasts but rapidly becomes unphosphorylated when the cells are induced to differentiate by growth factor removal. The change in the phosphorylated state of pRB, which coincides with the time that cells commit to terminal differentiation (Nadal-Ginard, 1978), is followed by cell fusion and the expression of muscle-specific genes in multinucleated myotubes (Gu et al., 1993). Previous studies indicated that pRB is required for both the growth arrest and myogenic functions of MyoD in the differentiation pathway of myotubes (Gu et al., 1993; Novitch et al., 1996; Schneider et al., 1994). Loss of pRB function in muscle cells has two prominent phenotypes. First, activation of terminal differentiation markers is dramatically reduced, and second, new DNA synthesis occurs in the nuclei of the rare differentiated myotubes. Because amplification and overexpression of MDM2 can inhibit terminal differentiation of muscle cells, a phenotype similar to loss of pRB, and MDM2 interacts with pRB physically, we predicted that overexpression of MDM2 will block the function of pRB in the derivative 14 hybrid. We tested whether MDM2 abolished the permanent cell cycle withdrawal in the hybrid. Our results showed that the hybrids have a low level of differentiation. The differentiated cells expressed MHC, but they did not fuse to form multinueated myotubes. The poorly differentiated cells did not reenter the cell cycle and synthesize DNA after growth factor stimulation. This indicates that pRB can still maintain the permanent cell cycle withdrawal in the differentiated myocytes. However, this does not mean that overexpression of MDM2 had no effect on the pRB function. Previous studies indicated that MyoD transactivation of muscle-specific genes to high levels requires pRB (Gu et al., 1993; Novitch et al., 1996) or the pRB related protein p107 (Schneider et al., 1994) in addition to maintenance of terminal differentiation. This may reflect two different pathways that pRB participates in muscle differentiation. These two pathways may depend on the level of pRB protein or the phosphorylation state of pRB. During the cell cycle,

hyperphosphorylation of pRB blocks its interaction with the transcription factor E2F1/DP1. E2F1/DP1 transactive genes required for S phase and promotes the cells to enter the cell cycle and proliferate. While at this stage, MDM2 may also interact with certain myogenic factors, such as the MEF2 family, and block its ability to activate the muscle differentiation pathway. When differentiation starts, pRB is unphosphorylated and binds to the E2F1/DP1 heterodimer to block their ability to activate target genes, consequently the cells stop proliferation and enter the G_0 state. The hypophosphorylated pRB also interacts with MDM2 and releases the myogenic factors to activate the differentiation of the muscle cells. This regulation may be controlled by the amount of MDM2. The inhibition of the entry into the cell cycle requires the phosphorylation of pRB protein. Therefore, overexpression of MDM2 does not have an effect on cell cycle control properties of pRB. The poorly differentiated myocytes can still remain in the G_0 state. Since MDM2 is overexpressed in the hybrids, it can interact both with pRB and the myogenic factors concurrently. pRB can not release the myogenic factors from binding to MDM2 and so the cells fail to differentiate. The results of our laboratory (data not shown) showed that forced expression of pRB in the hybrids containing amplified MDM2 results in enhanced muscle differentiation, which is consistent with this hypothesis.

3. Inhibition of muscle differentiation in the hybrids requires MEF2C

From the above experiments, inhibition of muscle differentiation by overexpression of MDM2 in the derivative 14 hybrid did not involve the cell cycle regulator p53. Because these hybrids lost their differentiation potential, we predicted that MDM2 might interfere with one of the myogenic factors or their coregulators.

The myogenic pathway is regulated by two families of transcription factors: the basic helix-loop-helix (bHLH) family and the myocyte enhancer binding factor-2 (MEF2) family. The four myogenic bHLH factors-MyoD, myogenin, Myf-5 and MRF4 function to establish the skeletal muscle phenotype. In addition to activating muscle structural genes,

these factors play a role in regulating the exit of myoblasts from the cell cycle. The myogenic bHLH factors collaborate with members of the MEF2 family of MADS-box transcription factors to activate muscle structural genes. This collaboration may require direct interactions between the DNA-binding domains of MEF2 and the myogenic bHLH factors, but only one of the factors needs to be bound to DNA. These interactions allow either factor to activate transcription through the other's DNA binding site (Molkentin et al., 1995).

Our experiments show that MDM2 does not interact with MyoD. However, forced expression of one of the MEF2 family member, MEF2C, restored MyoD activity in cells overexpressing MDM2 (Fiddler unpublished observation). This suggests that MDM2 is inhibiting MyoD activity indirectly, and that MEF2C is a likely target of MDM2 action. Our studies further show that MDM2 can bind to MEF2C in vitro by the MBP-binding assay, while MDM2 does not bind to MyoD in the same assay. To define the region of MEF2C required for MDM2 interaction, we also tested various MEF2C deletion mutants. The mutant 1-117, which lacks the transactivation domain of MEF2C, still retain the ability of binding to MBP-MDM2. This is in contrast to the MDM2 interactions with p53 and E2F1/DP1, the binding sites of these proteins are their activation domains. The mutant Δ40-57 has the c-terminal portion of the MADS box deleted, which has been shown to be important for dimerization and DNA binding. The mutant Δ58-85 lacks the MEF2 domain, which also plays a role in dimerization. These mutants still interact specifically with MBP-MDM2. While the deletion mutant Δ1-95, which does not have the MADS box and the MEF2 domain, totally abolished the ability of MEF2C to interact with MDM2. These data indicate that the N-terminus of MEF2C is the target for interaction with MDM2. Whether the MDM2 interaction involves both the MADS box and the MEF2C domain or just the region of amino acids 1-40 still needs to be determined.

Since MDM2 binds to the MADS box region of MEF2C and the MADS box region is conserved among the MADS-box family of transcription factors, we further tested whether MDM2 could interact with another MADS-box containing factor SRF, which has also been implicated in muscle differentiation (Groisman et al., 1996). Our results indicate that MDM2 also interacts with SRF. This result suggests that the interaction between MDM2 and the MADS-box region may be universal to the MADS-box proteins. MDM2 may interfere with these factors in their normal functional pathway.

Previous studies showed that specific residues within the MADS box are important for MADS box function. Mutation of specific residues abolished either the DNA binding or dimerization activity of MEF2C (Molkentin et al., 1996). Amino acid Lys-5 is critical for DNA binding activity and has little effect on dimerization, while mutations of amino acids Lys-4, Arg-15, Arg-17 and Lys-23 resulted in a partial loss of DNA binding activity without a loss in dimerization potential. Our results showed that all of these mutants still bound to the MDM2 in the pull down assay. This is consistent with our gel shift assay which indicates that MDM2 does not block MEF2C interacting with its DNA binding site. These observations also suggest that the interaction sites of these two proteins may involve a large region of MEF2C. The crystal structure of the interaction between MDM2 and p53 was resolved (Kussie et al., 1996). MDM2 forms a deep hydrophobic cleft in its 109-residue N-terminal domain on which the p53 peptide binds as an amphipathic α helix. The interface relies on the steric complementary between the MDM2 cleft and the hydrophobic face of the p53 α-helix and, in particular, on a triad of p53 amino acids-Phe^{19}, Trp^{23}, and Leu^{26}-which insert deep into the MDM2 cleft. In addition, the crystal structure of the MADS box region of SRF was also recently described (Pellegrini et al., 1995). The primary DNA-binding element of the MADS box is an antiparrallel coiled coil of two amphipathic α-helices, one from each subunit of a homodimer. This amphipathic α-helix corresponds to amino acids 12-39 of MEF2C (Pellegrini et al., 1995). One unusual aspect

of the MDM2-P53 complex is that the interface relies primarily on van der Waals contacts and the buried surface area is nearly all hydrophobic. The C-terminal end of the DNA binding α-helix of SRF does not make contacts with the DNA and would be available for protein-protein interactions (Pellegrini et al., 1995). Furthermore, this region of the MADS box is highly conserved among different members of the family, and the C-terminal end of this α-helix contains a pattern of hydrophobic amino acids similar to the hydrophobic amino acids important for p53 binding to MDM2 (compare KAYELSVLC of MEF2C with TFSDLWKLL of p53, hydrophobic amino acids are underlined). Therefore, mutation of this hydrophobic region of MEF2C may abolish the MDM2 binding of MEF2C.

By in vitro assay, we showed that MDM2 can bind to one of the MEF2 factors MEF2C, which has been shown to be an important coregulator of the myogenic pathway (Molkentin et al., 1995). This interaction may block the synergistic activity of bHLH myogenic factors and inhibit the differentiation of the myocytes. Although these two proteins can interact specifically in vitro, it is important to prove that these two protein can also interact by in vivo assay.

Since MDM2 amplification is a frequent event in rhabdomyosarcomas (Fiddler et al., 1996; Weber-Hall et al., 1996), the interaction between MDM2 and myogenic factors may be a common mechanism for the inhibition of differentiation. Understanding the mechanism of MDM2's function in the regulation of muscle differentiation may help to understand this disease.

Reference

Baker, S. J., Markowitz, S., Fearon, E. R., Wilson, J. K. V., and Vogelstein, B. (1990). Suppression of human colorectal carcinoma cell growth by wild-type p53. Science *249*, 912-915.

Barak, Y., Gottlieb, E., Juven-Gershon, T., and Oren, M. (1994). Regulation of *mdm2* expression by p53: alternative promoters produce transcripts with nonidentical translation potential. Genes Dev. *8*, 1739-1749.

Barak, Y., and Oren, M. (1992). Enhanced binding of a 95ld protein to p53 in cells undergoing p53-mediated growth arrest. EMBO J. *11*, 2115-2121.

Barr, F. G., Galili, N., holick, J., Biegel, J. A., Rovera, G., and Emanuel, B. S. (1993). Rearrangement of the PAX3 paired box gene in the paediatric solid tumor alveolar rhabdomyosarcoma. Nature Genetics *3*, 113-117.

Blackwell, K. T., and Weintraub, H. (1990). Differences and similarities in DNA-binding preferences of MyoD and E2A protein complexes revealed by binding site selection. Science *251*.

Boddy, M. N., Borden, K. L. B., and Freemont, P. S. (1994). The p53-associated protein MDM2 contains a newly characterized zinc-binding domain called the RING finger. Trends in Biochem. Sci. *19*, 19-20.

Cahilly-Snyder, L., Yang-Feng, T., Francke, U., and George, D. L. (1987). Molecular analysis and chromosomal mapping of amplified genes isolated from a transformed mouse 3T3 cell line. Somatic cell Mol. Genet. *13*, 235-244.

Chen, J., Marechal, V., and Levine, A. J. (1993). Mapping of the p53 and mdm-2 interaction domains. Mol. Cell. Biol. *13*, 4107-4114.

Chen, J., Wu, X., Lin, J., and Levine, A. J. (1996). mdm-2 inhibits the G1 arrest and apoptosis functions of the p53 tumor suppressor protein. Mol. Cell. Biol. *16*, 2445-2452.

Cheng, T.-C., Wallace, M. C., Merlie, J. P., and Olson, E. N. (1993). Separable regulatory elements governing myogenin transcription in mouse embryogenesis. Science *261*, 215-218.

Chin, K. V., Ueda, K., Pastan, I., and Gottesman, M. M. (1992). Modulation of activity of the promoter of the human MDR1 gene by Ras and p53. Science *255*, 459-462.

Clarke, A. R., Purdie, C. A., Harrison, D., J., Morris, R. G., Burd, C. C., Hooper, M. L., and Wyllie, A. H. (1993). Thymocyte apotosis induced by p53-dependent and independent pathways. Nature *362*, 849-852.

Cserjesi, P., and Olson, E. N. (1991). Myogenin induces the myocyte-specific enhancer binding factor MEF-2 independently of other muscle-specific gene products. Mol. Cell. Biol. *11*, 4854-4862.

Davis, R. J., D'Cruz, C. M., Lovell, M. A., Biegel, J. A., and Barr, F. G. (1994). Fusion of PAX7 to FKHR by the variant t(1;13)(p36;q14) translocation in alveolar rhabdomyosarcoma. Cancer Res. *54*, 2869-2872.

de Oca Luna, R. M., Wagner, D. S., and Lozano, G. (1995). Rescue of early embryonic in *mdm2*-deficient mice by deletion of *p53*. Nature *378*, 203-206.

Decaprio, J. A., Ludlow, J. W., Lynch, D., Furukawa, Y., Griffin, J., Piwnica-Worms, H., Huang, C.-M., and Livingston, D. M. (1989). The product of the retinoblastoma gene has properties of a cell cycle regulatory element. Cell *58*, 1085-1095.

Di-Leonardo, A., Linke, S. P., Clarkin, K., and Wahl, G. M. (1994). DNA damage triggers a prolonged p53-dependent G1 arrest and long-term induction of Cip1 in normal human fibroblasts. Genes Dev. *8*, 2540-2551.

Dias, P., Kumar, P., Marsden, H. B., Gattamaneni, H. R., Heighway, J., and Kumar, S. (1990a). N-myc gene is amplified in alveolar rhabdomyosarcomas (RMS) but not in embryonal RMS. Int. J. Cancer *45*, 593-596.

Dias, P., Parham, D., Shapiro, D. N., Webber, B. L., and Houghton, P. J. (1990). Myogenic regulatory protein (MyoD1) expression childhood solid tumors: diagnostic utility in rhabdomyosarcoma. Am. J. Pathol. *137*, 1283-1291.

Dyson, N., Howley, P. M., Munger, K., and Harlow, E. (1989). The human papillomavirus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. Science *243*, 934-937.

Edmondson, D., and Olson, E. N. (1993). Helix-loop-helix proteins as regulators of muscle-specific transcription (review). J. Biol. Chem. *268*, 755-758.Edmondson, D. G.,

Lyons, G. E., Martin, J. F., and Olson, E. N. (1994). *Mef2* gene expression marks the cardiac and skeletal muscle lineages during mouse embryogenesis. Development *120*, 1251-1263.

El-Deiry, W. S., Kern, S. E., Pietenpol, J. A., Kinzler, K. W., and Vogelstein, B. (1992). Deletion of a consensus binding site for p53. Nature Genet. *1*, 45-49.

Elledge, S. J. (1996). Cell cycle checkpoints: preventing an identity crisis. Science *274*, 1664-1672.

Endo, T., and Goto, S. (1992). Retinoblastoma gene product Rb accumulates during myogenic differentiation and is deinduced by the expression of SV40 large antigen. J. Biochem. *112*, 427-430.

Fakharzadeh, S. S., Trusko, S. P., and George, D. L. (1991). Tumorigenic potential associated with enhanced expression of a gene that is amplified in a mouse tumor cell line. EMBO J. *10*, 1565-1569.

Fiddler, T. A., Smith, L., Tapscott, S. J., and Thayer, M. J. (1996). Amplification of MDM2 inhibits MyoD-mediated myogenesis. Mol. Cell. Biol. *16*, 5048-5057.

Finlay, C. A. (1993). The mdm-2 oncogene can overcome wild type p53 suppression of transformed cell growth. Mol. Cell. Biol. *13*, 301-306.

Finlay, C. A., Hinds, P. W., and Levine, A. J. (1989). The p53 proto-oncogene can act as a suppressor of transformation. Cell *57*, 1083-1093.

Freemont, P. S. (1993). The RING finger: a novel protein sequence motif related to the

zinc finger. Anal. NY Acad. Sci. *684*, 174-192.

Fukasawa, K., Choi, T., Kuriyama, R., Rulong, S., and Vande Woude, G. (1996). Abnormal centrosome amplification in the absence of p53. Science *271*, 1744-1747.

Funk, W. D., Pak, D. T., Karas, R. H., Wright, W. E., and Shay, J. W. (1992). Transcriptionally active DNA-binding site for human p53 protein complexes. Mol. Cell. Biol. *12*, 2866-2871.

Gossett, L. A., Kelvin, D. J., Sternberg, E. A., and Olson, E. N. (1989). A new myocyte-specific enhancer-binding factor that recognizes a conserved element associated with multiple muscle-specific genes. Mol. Cell. Biol. *9*, 5022-5033.

Goulding, M. D., Chalepakis, G., Deutsch, U., Ersekius, J. R., and Gruss, P. (1991). Pax-3, a novel murine DNA binding protein expressed during early neurogenesis. EMBO J. *10*, 1135-1147.

Greenblatt, M., S. , Bennett, W. P., Hollstein, M., and Harris, C. C. (1994). Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. [Review]. Cancer Res. *54*, 4855-4878.

Groisman, R., Masutani, H., Leibovitch, M.-P., Robin, P., Soudant, I., Trouche, D., and Harel-Bellan, A. (1996). Physical interaction between the mitogen-responsive serum response factor and myogenic basic helix-loop-helix proteins. J. Biol. Chem. *271*, 5258-5264.

Gu, W., Schneider, J. W., Condorelli, G., Kaushal, S., Mahdavi, V., and Nadal-Ginard, B. (1993). Interaction of myogenic factors and the Retinoblastoma protein mediates muscle cell commitment and differentiation. Cell *72*, 309-324.

Haines, D. S., Landers, J. E., Engle, L. J., and George, D. L. (1994). Physical and functional interaction between wild-type p53 and mdm2 proteins. Mol. Cell. Biol. *14*, 1171-1178.

Halevy, O., Novitch, B. G., Spicer, D. B., Skapek, S. X., Rhee, J., Hannon, G. J., Beach, D., and Lassar, A. B. (1995). Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD [see comments]. Science *267*, 1018-1021.

Hazelton, B. J., Houghton, J. A., Parham, D. M., Douglass, E. C., Torrance, P. M., Holt, H., and Houghton, P. J. (1987). Characterization of cell lines derived from Xenografts of childhood rhabdomyosarcoma. Cancer Res. *47*, 4501-4507.

Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C. C. (1991). p53 mutations in human cancers. Science *252*, 49-53.

Horn, R. C., and Enterline, H. F. (1958). Rhabdomyosarcoma: A clinic-pathological study and classification of 39 cases. Cancer *11*, 181.

Iavarone, A., Garg, P., Lasorella, A., Hsu, J., and Israel, A. (1994). The helix-loop-helix protein Id-2 enhances cell proliferation and binds to the retinoblastoma protein. Genes Dev. *8*, 1270-1284.

Johnson, W. W., Jurand, J., and Hiramto, R. (1965). Immunohistologic studies of tumor containing myosin. Am. J. Pathol. *47*, 1139-1155.

Jones, S. N., Roe, A. E., Donehower, L. A., and Bradley, A. (1995). Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53. Nature *378*, 206-208.

Jostes, B., Walther, C., and Gruss, P. (1991). The murine paired box gene, pax7, is expressed specifically during the development of the nervous and muscular system. Mech. Dev. *33*, 27-37.

Juven, T., Barak, Y., Zauberman, A., George, D. L., and Oren, M. (1993). Wild type p53 can mediate sequence-specific transactivation of an internal promoter within the *mdm2* gene. Oncogene *8*, 3411-3416.

Kaushal, S., Schneider, J. W., Nadal-Gimard, B., and Mahdavi, V. (1994). Activation of the myogenic lineage by MEF2A, a factor that induces and cooperates with MyoD. Science *266*, 1236-1240.

Kern, S. E., Kinzler, K. W., Bruskin, A., Jarosz, D., Friedman, P., Prives, C., and Vogelstein, B. (1991). Identification of p53 as a sequence-specific DNA-binding protein. Science *252*, 827-830.

Kern, S. E., Pietenpol, J. A., Thiagalingam, S., Seymour, A., Kinzler, K. W., and Vogelstein, B. (1992). Oncogenic forms of p53 inhibits p53-regulated gene expression. Science *256*, 827-830.

Kuerbitz, S. J., Plunkett, B. S., Walsh, W. V., and Kastan, M. B. (1992). Wild-type p53 is a cell cycle checkpoint determinant following irradiation. Proc. Natl. Acad. Sci. USA *89*, 7491-7495.

Kussie, P. H., Gorina, S., Marechal, V., Elenbaas, B., Moreau, J., Levine, A. J., and Pavletich, N. P. (1996). Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain. Science *274*, 948-953.

Levine, A. J., Momand, J., and Finlay, C. A. (1991). The p53 tumor suppressor gene. Nature *351*, 453-455.

Li, H., and Capetanaki, Y. (1991). An E box in the desmin promoter cooperates with the E box and MEF-2 sites of a distal enhancer to direct muscle-specific transcription. EMBO J. *13*, 3580-3589.

Livingstone, L. R., White, A., Sprouse, J., Livanos, E., Jacks, T., and Tlsty, T. D. (1992). Altered cell cycle arrest and gene amplification potential accompany loss of wild-type p53. Cell *70*, 923-935.

Lowe, S. W., Ruley, H. E., Jacks, T., and Housman, D. E. (1993). p53 dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell *74*, 957-967.

Macleod, K. F., Sherry, N., Hannon, G., Beach, D., Tokino, T., Kinzler, K., Vogelstein, B., and Jacks, T. (1995). p53-dependent and independent expression of p21 during cell growth, differentiation, and DNA damage. Genes Dev. *9*, 935-944.

Martelli, F., Cenciarelli, C., Santarelli, G., Polikar, B., Felsani, A., and Caruso, M. (1994). MyoD induces retinoblastoma gene expression during myogenic differentiation. Oncogene *9*, 3579-3590.

Martin, J. F., Cshwarz, J. J., and Olson, E. N. (1993). Myocyte enhancer factor (MEF)2C: a tissue-restricted member of the MEF-2 family of transcription factors. Proc. Natl. Acad. Sci. USA. *90*.

Martin, K., Trouche, D., Hagemeier, C., Sorensen, T. S., La Thangue, N. B., and Kouzarides, T. (1995). Stimulation of E2F1/Dp1 translational activity by MDM2 oncoprotein. Nature *375*, 691-694.

McDermott, J. C., Cardoso, M. C., Yu, Y.-T., Andres, V., Leifer, D., Krainc, D., Lipton, S. A., and Nadal-Ginard, B. (1993). hMEF2C gene encodes skeletal muscle- and Brain-specific transcription factors. Mol. Cell. Biol. *13*, 2564-2577.

Megeney, L. A., and Rudnicki, M. A. (1995). Determination versus differentiation and the MyoD family of transcription factors. Biochem. Cell. Biol. *73*, 723-732.

Molkentin, J. D., Black, B. L., Martin, J. F., and Olson, E. N. (1995). Cooperative activation of muscle gene expression by MEF2 and myogenic bHLH proteins. Cell *83*, 1125-1136.

Molkentin, J. D., Black, B. L., Martin, J. F., and Olson, E. N. (1996). Mutational analysis of the DNA binding, dimerization, and transcriptional activation domains of MEF2C. Mol. Cell. Biol. 16, 2627-2636.

Momand, J., Zambetti, G. P., Olson, D., George, D. L., and Levine, A. J. (1992). The mdm2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. Cell *69*, 1237-1245.

Muller, R. (1995). Transcriptional regulation during the mammalian cell cycle. Tren. in Genet. *11*, 173-178.

Nadal-Ginard, B. (1978). Commitment, fusion, and biochemical differentiation of a myogenic cell line in the absence of DNA synthesis. Cell *15*, 855-864.

Naidus, P. S., Ludolph, D. C., To, R. Q., Hinterberger, T. J., and Konoeczny, S. F. (1995). Myogenin and MEF2 function synergistically to activate the MRF4 promoter during myogenesis. Mol. Cell. Biol. *15*, 2707-2718.

Newsham, I., Claussen, U., Ludecke, H. J., Mason, M., Senger, G., Horsthemke, B., and Cavenee, W. (1991). Microdissection of chromosome band 11p15.5: characterization of probes mapping distal to the HBBC locus. Genes Chromosome Cancer *3*, 108-116.

Novitch, B. G., Mulligan, G. J., Jacks, T., and Lassar, A. B. (1996). Skeletal muscle cells lacking the Retinoblastoma protein display defects in muscle gene expression and accumulate in S and G_2 phases of the cell cycle. J. Cell Biol. *135*, 441-456.

Oliner, J. D., Kinzler, K. W., Meltzer, P. S., George, D. L., and Vogelstein, B. (1992). Amplification of a gene encoding a p53-associated protein in human sarcomas. Nature *358*, 80-83.

Oliner, J. D., Pietenpol, J. A., Thiagalingam, S., Gyuris, J., Kinzler, K. W., and Vogelstein, B. (1993). Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53. Nature *362*, 857-860.

Olson, D. C., Marechal, V., Momand, J., Chen, J., Romocki, C., and Levine, A. J. (1993). Identification and characterization of multiple mdm-2 proteins and mdm2-p53 protein complexes. Oncogene *8*, 2353-2360.

Olson, E. N. (1992). Interplay between proliferation and differentiation within the myogenic lineage. Dev. Biol. *154*, 261-272.

Olson, E. N., Perry, M., and Schulz, R. A. (1995). Regulation of muscle differentiation by the MEF2 family of MADS box transcription factors (review). Dev. Biol. *172*, 2-14.

Parker, S. B., Eichele, G., Zhang, P., Rawls, A., Sands, A. T., Bradley, A., Olson, E. N., Harper, J. W., and Elledge, S. J. (1995). p53-independent expression of p21 Cip1 in muscle and other terminally differentiating cells [see comments]. Science *267*, 2024-1027.

Pellegrini, L., Tan, S., and Richmond, T. J. (1995). Structure of serum response factor core bound to DNA [see comments]. Nature *376*, 490-498.

Pollock, R., and Treisman, R. (1991). Human SRF-related proteins: DNA-binding properties and potential regulatory targets. Genes Dev. *5*, 2327-2341.

Reifenberger, G., Reifenberger, j., Ichimura, K., Meltzer, P. S., and Collins, P. V. (1994). Amplification of multiple genes from chromosomal 12q13-14 in human malignant gliomas: preliminary mapping of the amplicons shows preferential involvement of CDK4, SAS, and MDM2. Cancer Res. *54*, 4299-4303.

Roberts, P., Browne, C. F., Lewis, I. J., Bailey, C. C., Spicer, R. D., Williams, J., and Batcup, G. (1992). 12q13 abnormality in rhabdomyosarcoma. Cancer Genet Cytogent *60*, 135-140.

Schneider, J. W., Gu, W., Zhu, L., Mahdavi, V., and Nadal-Ginard, B. (1994). Reversal of terminal differentiation mediated by p107 in Rb-/- muscle cells. Science *264*, 1467-1471.

Scrable, H. J., Johnson, D. K., Rinchik, E. M., and Cavenee, W. K. (1990). Rhabdomyosarcoma-associated locus and MYOD1 are syntenic but separate loci on the short arm of human chromosome 11. Proc. Natl. Acad. Sci. USA *87*, 2182-2186.

Seto, E., Usheva, A., Zambetti, G. P., Momand, J., Horokoshi, N., Weinmann, R., Levine, A. J., and Shenk, T. (1992). Wild-type p53 binds to the TATA-binding protein and represses transcription. Proc. Natl. Acad. Sci. USA *89*, 12028-12032.

Shapiro, D. N., Sulett, J. E., Li, B., Downing, J. R., and Naeve, C. W. (1993). Fusion of PAX3 to a member of the forkhead family of transcriptional factors in human alveolar rhabdomyosarcoma. Cancer Res. *53*, 5108-5112.

Shaulsky, G., goldfinger, N., Peled, A., and Rotter, V. (1991). Involvement of wild-type p53 in pre-B cell differentiation in vitro. Proc. Natl. Acad. Sci. USA *88*, 8982-8986.

Shaw, P., Bovey, R., Tardy, S., Sahli, R., Sordat, B., and Costa, J. (1992). Involvement of wild-type p53 in a human colon tumor-derived cell line. Proc. Natl. Acad. Sci. USA *89*, 4495-4499.

Shirakata, M., Friedman, F. K., Wei, Q., and Paterson, B. M. (1993). Dimerization specificity of myogenic helix-loop-helix DNA-binding factors directed by nonconserved

hydrophobic residues. Genes Dev. *7*, 2456-2470.

Shore, P., and Sharrocks, A. D. (1995). The MADS-box family of transcription factors. Eur. J. Biochem. *229*, 1-13.

Subler, M. A., Martin, D. W., and Deb, S. (1992). Inhibition of viral and cellular promoters by human wild-type p53. J. Virol. *66*, 4757-4762.

Suijkerbuijk, R. F., Olde-Weghuis, D. E., Van-den-Berg, M., Pedeutour, F., Forus, A., Myklebost, O., Giler, C., Turc-Carel, C., and Geurts-van-Kessel, A. (1994). Comparative genomic hybridization as a tool to define two distinct chromosome 12-derived amplification units in well-differentiated liposarcomas. Genes Chromosomes Cancer *9*, 292-295.

Tapscott, S. J., Thayer, M. J., and Weintraub, H. (1993). Deficiency in rhabdomyosarcomas of a factor required for MyoD activity and myogenesis. Science *259*, 1450-1453.

Volgelstein, B., and Kinzler, K. W. (1992). p53 function and dysfunction. Cell *70*, 523-526.

Wang-Wuu, S., Soukup, S., Ballard, E., Gpttwals, B., and Lampkin, B. (1988). Chromosomal analysis of sixteen human rhabdomyosarcomas. Cancer Res. *48*, 983-987.

Weber-Hall, S., Anderson, J., McManus, A., Abe, S., Nojima, T., Pinkerton, R., Pritchard-Jones, K., and Shipley, J. (1996). Gaines, losses, and amplification of genomic materials in rhabdomyosarcoma analyzed by comparative genomic hybridization. Cancer Res. *56*, 3220-3224.

Weinberg, R. (1995). The retinoblastoma protein and cell cycle control. Cell *81*, 323-330.

Weintraub, H., Dwarki, V. J., Verma, I., Davis, R., Hollenberg, S., Snider, L., Lassar, A., and Tapscott, S. J. (1991). Muscle-specific transcriptional activation by MyoD. Genes Dev. *5*, 1377-1386.

Weintraub, H., Tapscott, S. J., Davis, R. L., Thayer, M. J., Adam, M. A., Lassar, A. B., and Miller, A. D. (1989). Activation of muscle-specific genes in pigment, nerve, fat, liver, and fibroblast cell lines by forced expression of MyoD. Proc. Natl. Acad. Sci. USA *86*, 5434-5438.

Wu, X., Bayle, H. J., Olson, D., and Levine, A. J. (1993). The p53-mdm-2 autoregulatory feedback loop. Genes Dev. *7*, 1126-1132.

Xiao, Z.-X., Chen, J., Levine, A., Modjtahedi, N., Xing, J., Sellers, W. R., and Livingston, D. M. (1995). Interaction between the retinoblastoma protein and the oncoprotein MDM2. Nature *375*, 694-698.

Yin, Y., Tainsky, M. A., Bischoff, F. Z., Strong, L. C., and Wahl, G. M. (1992). Wild-type p53 restores cell cycle control and inhibits gene amplification in cells with mutant p53 alleles. Cell *70*, 937-948.

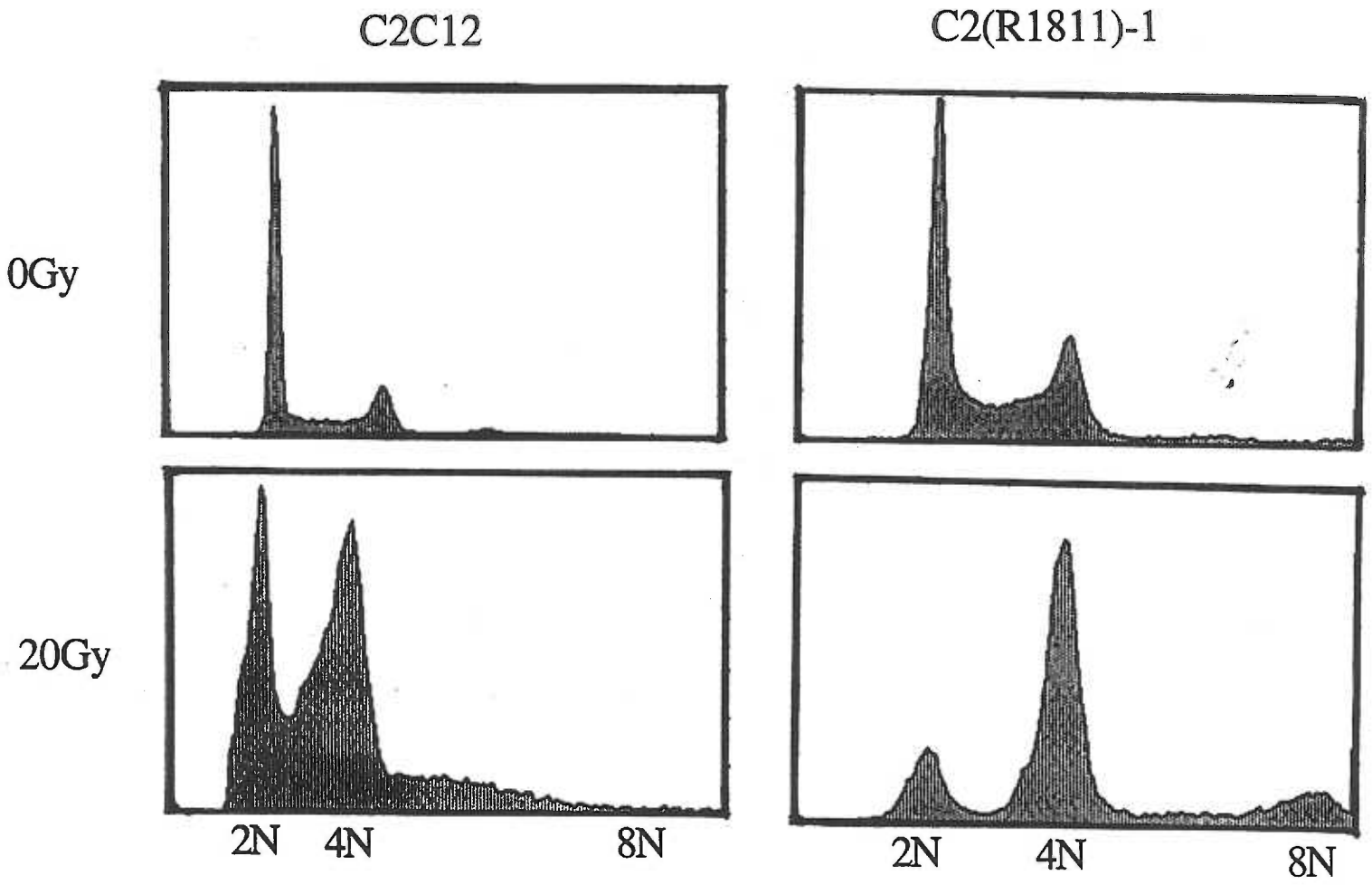
Yin, Y., Tainsky, M. A., Bischoff, F. Z., Strong, L. C., and Wahl, G. M. (1992). Wild-type p53 restores cell cycle control and inhibits gene amplification in cells with mutant p53 alleles. Cell *70*, 937-948.

Yun, K., and Wold, B. (1996). Skeletal muscle determination and differentiation: story of a core regulatory network and its context. Current opinion in Cell Biology *8*, 877-889.

Zambetti, G. P., Bargometti, J., Walker, K., Prives, C., and Levine, A. J. (1992). Wild-type p53 mediates positive regulation of gene expression through a specific DNA sequence element. Genes. Dev. *6*, 1143-1152.

Figure 2. Flow cytometry analysis of cells after 0 or 20 Gy of γ-irradiation.

G1 arrest of the MDM2 overexpressed hybrid C2(R1811)-1. Parental C2C12 and the hybrid C2(R1811)-1 were irradiated with 0 Gy or 20 Gy IR and harvested after 17 hours. The cells were stained with propidium iodide and analyzed by FACS analysis. The horizontal axis shows the DNA content of the cell. The vertical axis shows the number of cells at each stage of the cell cycle (G1, S, G2).


C2C12
C2(R1811)-1
0Gy
20Gy
2N
4N
8N
2N
4N
8N

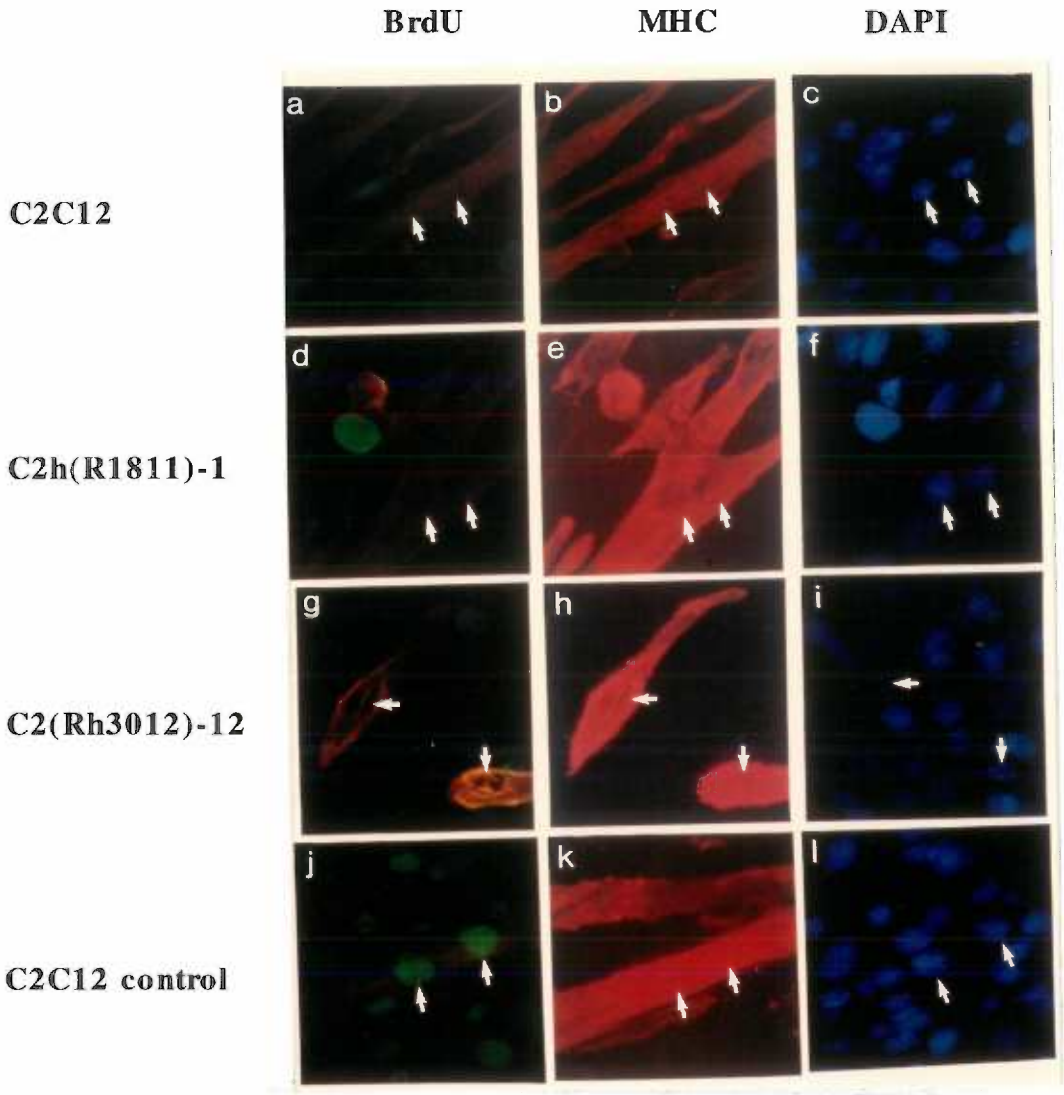


Figure 3. Immunostaining of cells with anti-MHC and anti-BrdU antibodies for cell cycle analysis.

The cells were in the differentiating medium for 72 hours and refed with medium containing high serum and 10 μM BrdU for 24 hours. Cells were then fixed, permealized, and stained for MHC (red), BrdU (green) and DAPI (blue). The fourth row is the control that put BrdU before the differentiation, so the myotubes have positive BrdU staining.

Figure 4. Ratio of BrdU incorporation in Nuclei of "differentiated cells"

Cell Line	C2C12		C2(R1811)-1		C2(Rh30)-12	
Growth Medium and BrdU	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr
Differentiated	3.6	2.9	0.9	3.5	2.0	2.0
Non-Differentiated	35	76	31	60	18	80

% BrdU positive

Figure 5. MBP-MDM2 pull down assay.

Full length MDM2 was fused in frame to the E.Coli maltose binding protein (MBP). MEF2C, p53 and MyoD were translated in vitro in the presence of ^{35}S methionine and incubated with either MBP-MDM2 coated maltose resin or MBP MDM2 coated maltose resin alone. Bound proteins were isolated by the resin and resolved bound proteins by SDS-PAGE. The figure shows the representative lanes of the SDS-PAGE gel result.

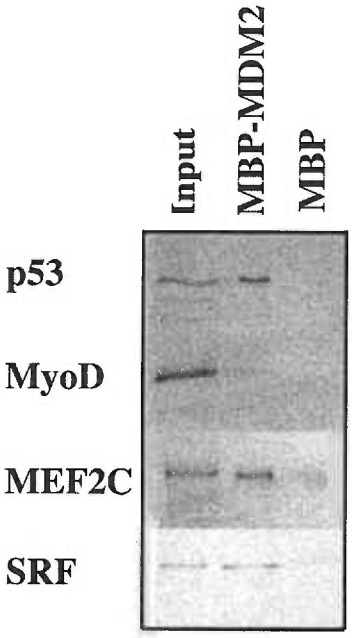
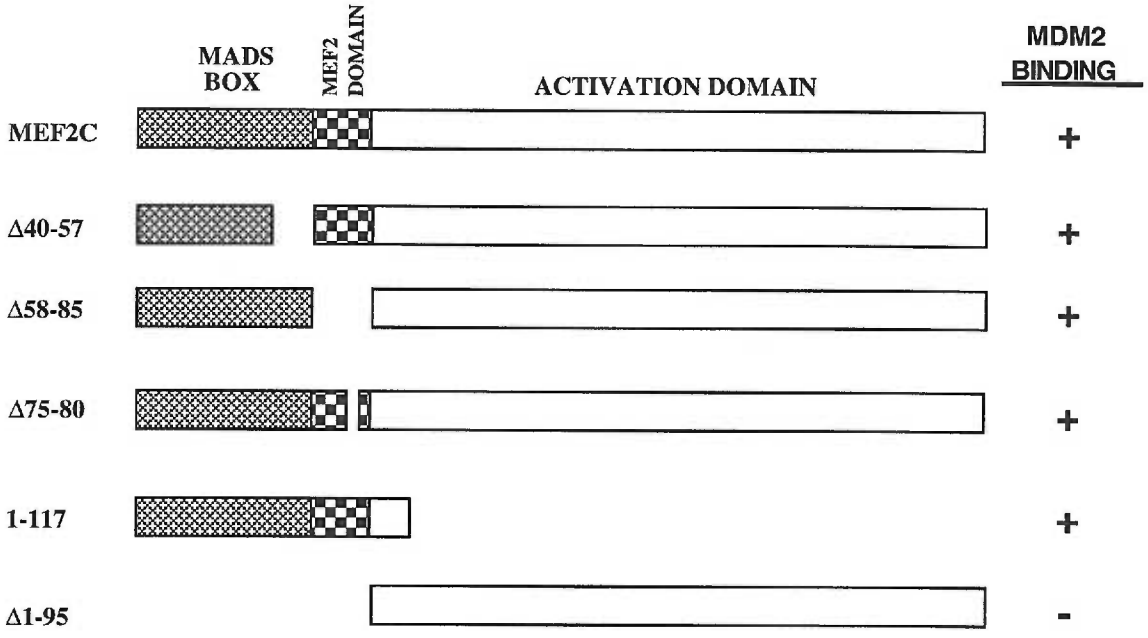
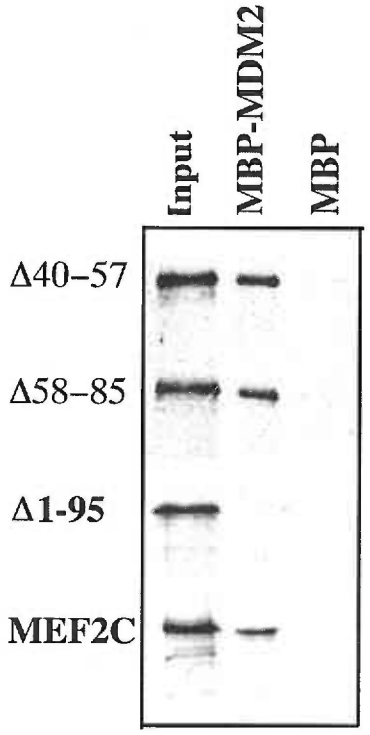

Input
MBP-MDM2
MBP
p53
MyoD
MEF2C
SRF

Figure 6. A) MEF2C deletion mutants, showing the location of the MADS box, MEF2 domain and the activation domain. B) MBP-MDM2 pull down assay on deletion mutants of MEF2C: Δ40-57, Δ58-85, and Δ1-95.

In vitro translated proteins, labeled with ^{35}S methionine, were incubated with either MBP-MDM2 coated maltose resin or MBP-MDM2 coated maltose resin alone, pull down by the resin and separated by SDS-PAGE. The input lane shows the amount of each protein added to the reaction. The figure shows the representative lanes of the SDS-PAGE gel result.


MADS BOX
MEF2 DOMAIN
ACTIVATION DOMAIN
MDM2 BINDING
MEF2C
+
Δ40-57
+
Δ58-85
+
Δ75-80
+
1-117
+
Δ1-95
-

A



B

Figure 7. MBP-MDM2 pull down assay on point mutants of MEF2C: K4I, K5I, R15L, R17L, and K23T.

In vitro translated proteins, labeled with ^{35}S methionine, were incubated with either MBP-MDM2 coated maltose resin or MBP-MDM2 coated maltose resin alone, pull down by the resin and separated by SDS-PAGE. The input lane shows the amount of each protein added to the reaction. The figure shows the representative lanes of the SDS-PAGE gel result.

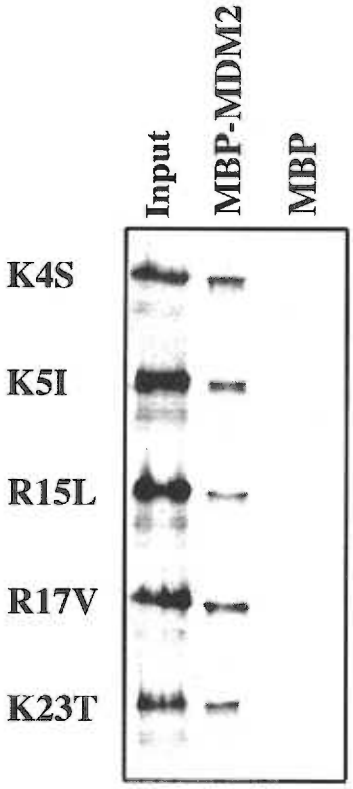
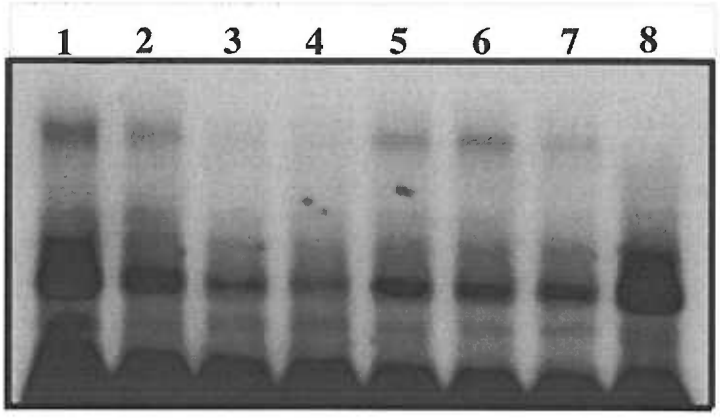
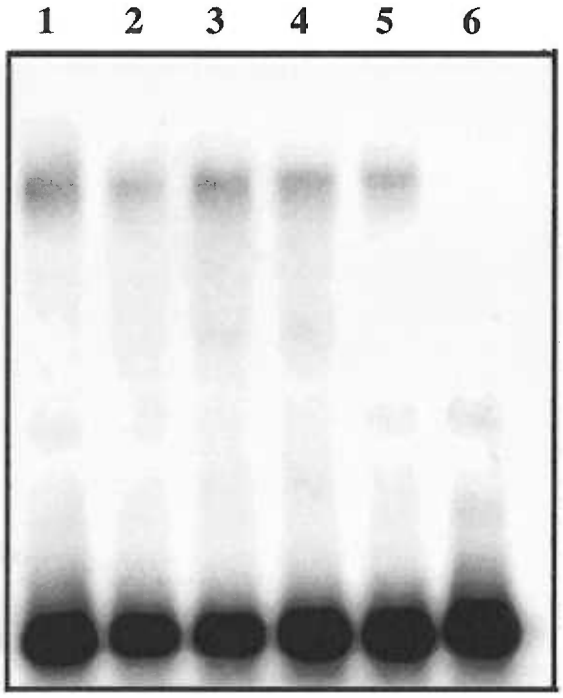

Input
MBP-MDM2
MBP
K4S
K5I
R15L
R17V
K23T

Figure 8. Electrophoresis mobility shift assay of MEF2C binding to its target DNA sequence in the presence of MDM2.

(A) This figure shows the specific MEF2C shifted band. Multimerized ^{32}P-labelled MEF2C DNA binding sequence was incubated with in vitro translated MEF2C protein in the presence of increasing amount of unlabelled MEF2C DNA binding site or MyoD DNA binding site. Lane 1 shows the reaction with MEF2C protein. Lane 8 shows the reaction without adding MEF2C. Lanes 2-4 show the reactions with 0, 10x, 100x unlabelled MEF2C DNA-binding site. Lanes 5-7 show the reactions with 0, 10x, 100x unlabelled nonspecific oligonucleotide sequences. (B) Multimerized ^{32}P-labelled MEF2C DNA binding sequence was incubated with in vitro translated MEF2C protein in the presence of increasing amount of MDM2 protein. The reaction was separated by electrophoresis through 2% low melting-point agarose gel. Lane 6 shows the reaction without adding MEF2C. Lane 1 is the control with MEF2C, but in the absent of MDM2. Lanes 2-5 shows the binding of MEF2C to its DNA site with increasing amount of MDM2 (from 1:1 ratio to 1:8).



A



B