MEF2: a transcriptional target for signaling pathways controlling skeletal muscle growth and differentiation
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Skeletal muscle development involves a multistep pathway in which mesodermal precursor cells are selected, in response to inductive cues, to form myoblasts that later withdraw from the cell cycle and differentiate. The transcriptional circuitry controlling muscle differentiation is intimately linked to the cell cycle machinery, such that muscle differentiation genes do not become transcribed until myoblasts have exited the cell cycle. Members of the MyoD and MEF2 families of transcription factors associate combinatorially to control myoblast specification, transcriptional activation, and proliferation. Recent studies have revealed multiple signaling systems that stimulate and inhibit myogenesis by altering MEF2 phosphorylation and its association with other transcriptional cofactors.

Abbreviations
bHLH basic helix-loop-helix
MEF2 myocyte enhancer factor 2
MKP MAP kinase phosphatase
NFAT nuclear factors of activated T cells
Rb retinoblastoma
TGF-β transforming growth factor β

Introduction
Skeletal muscle has become a model for understanding many fundamental principles of development, including mechanisms for cell fate specification, differentiation, morphogenesis and the antagonism between growth and differentiation. Many of the steps involved in the genesis of the muscle differentiation pathway are defective in the second step, suggesting that the critical transcriptional circuitry controlling muscle differentiation is intimately linked to the cell cycle machinery, such that muscle differentiation genes do not become transcribed until myoblasts have exited the cell cycle. Members of the MyoD and MEF2 families of transcription factors associate combinatorially to control myoblast specification, transcriptional activation and proliferation. Recent studies have revealed multiple signaling systems that stimulate and inhibit myogenesis by altering MEF2 phosphorylation and its association with other transcriptional cofactors.


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In addition to their roles in the transcriptional activation of muscle-specific genes, the MyoD and MEF2 families serve as end points for the diverse intracellular signaling pathways that control myogenesis through modulation of the functions and the expression of these factors. These myogenic transcription factors also engage the cell cycle machinery to regulate the decision of myoblasts to divide or differentiate. Here, we review recent studies that further refine our understanding of the transcriptional circuits and signaling systems that control skeletal myogenesis and the central role of MEF2 factors in these developmental processes.

The partnership of MEF2 and MyoD
Previous studies have demonstrated that muscle-specific gene expression and myogenesis are regulated by combinatorial associations between myogenic bHLH proteins and MEF2 factors, and that the DNA-binding domains of these factors mediate their interactions [3]. MEF2 factors can only cooperate with heterodimers of myogenic bHLH proteins and E proteins, such as E12 and E47, not with E protein homodimers, which bind the same DNA sequence as MyoD–E12 heterodimers. An alanine and a threonine in the center of the basic regions of all members of the MyoD family are essential for muscle gene activation. Replacement of these residues with those from the corresponding region of the ubiquitous E protein E12 eliminates myogenic activity without affecting DNA binding, suggesting that these residues mediate interaction with an essential myogenic cofactor. Consistent with this notion, MEF2 factors are unable to co-operate with MyoD mutants lacking the essential alanine and threonine to activate myogenesis, even though MEF2 factors were recently (and unexpectedly) shown to interact with this type of MyoD mutant [4]. These findings demonstrate that the myogenic bHLH–MEF2 interaction can be uncoupled from transcriptional activation and suggest that muscle gene activation by MyoD and MEF2 involves a two-step process in which these factors must first interact and subsequently adopt a transcriptionally active conformation. MyoD basic domain mutants can interact with MEF2, but are defective in the second step, suggesting that the critical alanine and threonine are required to transmit a transcriptional activation signal in conjunction with MEF2.

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The mechanism by which the MyoD basic domain activates myogenesis remains to be defined. We favor the possibility that it specifically recognizes essential downstream target genes in the myogenic pathway and, although
basic domain mutants may bind the same DNA sequences as wild-type MyoD in vitro and activate transcription through E-boxes in transiently transfected reporter genes, they may be unable to activate the critical genes in vivo in the context of native chromatin. This interpretation is consistent with recent studies demonstrating that MyoD initiates chromatin remodeling when bound to endogenous muscle-specific control regions, whereas MyoD basic domain mutants do not [5]. MyoD has also been shown to interact with histone acetyltransferases and deacetylases to activate and repress transcription, respectively [6–8], further illustrating the importance of interactions between myogenic bHLH proteins and chromatin associated factors in activating myogenesis.

Multiple partners for MEF2

Although MEF2 was initially identified as a muscle-restricted transcription factor that bound a conserved A/T-rich element in numerous muscle-specific genes, it is also expressed at high levels in neurons, as well as in T cells and fibroblasts. In each of these cell types, an important function of MEF2 is to transmit signals from the cell membrane to downstream immediate early genes and stress-response genes. The selection of genes regulated by MEF2 is determined by phosphorylation-dependent activation of pre-existing MEF2 protein and by combinatorial associations with other transcription factors. In addition to interacting with members of the MyoD family, MEF2 factors have recently been shown to establish protein–protein interactions with several other transcription factors.

Transcriptional activation of the myoglobin promoter in striated muscle requires binding sites for both MEF2 and Sp1. MEF2 and Sp1 interact directly to synergistically activate the myoglobin promoter of artificial promoters containing binding sites for both factors [9]. Activation of the N-methyl-D-aspartate (NMDA) receptor subunit 1 (NR1) gene in neurons requires MEF2C and Sp1 [10]. Synergy between the two factors is mediated by their direct interaction and activation requires only the DNA-binding domain of MEF2C. MEF2 has also been shown to cooperate with the POU homeodomain protein Oct-1, which is expressed in a wide range of cell types, to activate the Hb myosin heavy chain (HBMHC) gene [11].

Detection of MEF2 transcriptional activity in vivo using MEF2 sensor mice

In many cell types, there is a disparity between the expression of MEF2 mRNA, the level of MEF2 protein and MEF2 transcriptional activity. In an effort to identify cell types in vivo in which MEF2 protein was transcriptionally active, we created transgenic mice, referred to as MEF2 sensor mice, that harbor a lacZ transgene under the control of three tandem copies of the MEF2 consensus DNA binding site [12•]. During early embryogenesis, these mice expressed lacZ in developing cardiac, skeletal and smooth muscle cells, consistent with the demonstrated role of MEF2 in myocyte differentiation (Figure 1). After birth, however, transgene expression is downregulated in these muscle cell types, despite the presence of high levels of MEF2 protein. These findings suggest that MEF2 factors are silenced through a post-translational mechanism in postnatal muscle cells. As discussed below, several recent studies have revealed important roles for MEF2 factors as end points for intracellular signaling pathways that stimulate and inhibit myogenesis. Using MEF2 sensor mice, it should be possible to further identify the signals that control MEF2 activity in vivo and to identify roles for MEF2 activation in adult muscle cells.

Control of MEF2 activity by transforming growth factor-β

Previous studies have shown that myogenic bHLH factors are inactive in cells exposed to transforming growth factor β (TGF-β), but they retain the ability to bind DNA under these conditions, suggesting that an essential myogenic cofactor might be the target of negative regulation by the TGF-β signaling pathway. De Angelis et al. [13•] demonstrated that MEF2 undergoes translocation from the nucleus to the cytoplasm in myoblasts stimulated with TGF-β and this translocation can be overridden by a MEF2 mutant containing an SV40 nuclear localization signal. This TGF-β-dependent blockade to MEF2 function may be important in regulating the timing of myogenesis in vivo, but this remains to be determined. The mechanism for this
translocation and the cytoplasmic proteins that might serve as anchors for MEF2 also remain to be identified.

**Notch-dependent signaling to MEF2C**

The transmembrane receptor Notch and its ligand Delta in *Drosophila*, and Jagged and serrate in vertebrates inhibit myogenesis. Previous studies have shown that Notch can block the ability of MyoD to activate myogenesis, suggesting that Notch activates a signaling system that targets MyoD directly or a cofactor for MyoD. The inhibitory activity of Notch appears to be directed at the MyoD basic region, but Notch does not alter MyoD DNA-binding activity. Given that MEF2 proteins interact with the MyoD basic region to co-operatively activate transcription, it is conceivable that Notch may exert its inhibitory effects on members of the MEF2 family. Indeed, a constitutively active form of Notch was recently shown to inhibit the activity of MEF2C through a direct interaction with a region immediately adjacent to the MEF2 domain, thus blocking its ability to bind DNA. Co-operative interaction between MEF2 and MyoD or myogenin was also prevented by Notch, providing a mechanism for myogenic repression [14].

**Regulation of MEF2 activity by MAP kinase signaling**

Recent studies have demonstrated that MAP kinase signaling controls several steps in the myogenic pathway [15,16] (Figure 2). Ectopic expression of the broad specificity MAP kinase phosphatase MKP-1 in C2C12 myoblasts is sufficient to interfere with the inhibitory influence of serum mitogens on myogenesis and allow precocious differentiation [15]. The target for MKP-1 action in proliferating myoblasts appears to be the MAP kinase p42Erk2, which is required for cyclin D1 expression. Upon inactivation of p42Erk2 by MKP-1, cyclin D1 expression declines and myoblasts exit the cell cycle. MKP-1 expression is down-regulated in myotubes during later stages of muscle differentiation, raising the possibility that MAP kinase activity may also play a role in maintenance of the muscle differentiation program. Consistent with this notion, ectopic expression of MKP-1 in differentiating muscle cells prevents terminal differentiation and formation of multinucleate myotubes. The kinase activity of the MAP kinase p38 is induced during differentiation of L6 myoblasts and the p38 inhibitor SB202190 inhibits myogenesis and blocks expression of MyoD and MEF2 factors [16]. Moreover, ectopic expression of p38 or the p38 activator MKK6 stimulates the ability of MyoD to convert fibroblasts into muscle cells. Thus, MAP kinases appear to play dual roles in repressing and activating muscle gene expression during early and late stages, respectively, of myogenesis.

What are the targets of MAP kinases in muscle cells? MEF2 proteins have been shown to interact directly with p38 and of MAP kinase ERK5/BMK1, and to be phosphorylated by both kinases [17–23,24**]. Conserved MAP kinase phosphorylation sites are found in the transcription activation domains of multiple MEF2 proteins. Recent studies have identified specific docking sites in MEF2A and MEF2C for different MAP kinases [24**], thereby providing a mechanism for selective targeting of specific MEF2 isoforms by different MAP kinases. Phosphorylation of these sites stimulates transcriptional activity of MEF2 factors [24**], thereby enhancing interaction of MEF2 proteins with the transcriptional machinery. Phosphorylation could also be...
required for either recruitment of an essential transcriptional cofactor or release from a repressor.

In addition to their roles as regulators of muscle-specific genes and effectors for myogenic bHLH proteins, MEF2 proteins activate several growth- and stress-induced genes which are also dependent on the MAP kinase pathway. It seems paradoxical that MEF2 factors regulate both growth-induced and muscle-specific genes, at least in part, by different mechanisms. A critical question is how they interact with other transcription factors, and it seems likely that the spectrum of MEF2-dependent genes activated in a particular cell depends on the availability of other promoter-specific factors.

At the interface of muscle transcription and cell cycle control

In addition to their well-characterized roles in muscle cell specification and differentiation, members of the MyoD family of bHLH factors are intimately involved in controlling cell cycle progression and responsiveness of myoblasts to extracellular signals. Withdrawal from the cell cycle is a prerequisite for activation of the skeletal muscle differentiation program. The decision of a myoblast to divide or differentiate is controlled by the balance of positive and negative cell cycle regulators [25]. The cyclin-dependent kinases (Cdks) regulate cell cycle transitions by complexing with specific cyclins that control phosphorylation of the retinoblastoma (Rb) protein. When Rb becomes dephosphorylated, it evokes growth arrest in the G1 phase of the cell cycle by inactivating E2F. Previous studies have demonstrated that Rb is required for irreversible withdrawal of myoblasts from the cell cycle in response to MyoD and for transcriptional activation of a muscle-specific program of gene expression. Withdrawal of myoblasts from the cell cycle is required for either recruitment of an essential transcriptional cofactor or release from a repressor. Alternatively, they could activate another factor in conjunction with Rb that is required by myogenin to activate the differentiation program.

An interesting clue to the mechanism underlying the requirement of Rb for myoblast differentiation comes from studies of Lassar and colleagues [29••], who showed that MEF2C is functionally inactive in Rb–/– myoblasts and, as a consequence, muscle gene activation is perturbed. DNA-binding activity of MEF2C is normal in Rb–/– myoblasts, but the carboxyl-terminal transcription activation domain is inactive. The requirement of Rb for activation of MEF2C can be partially bypassed by fusing MEF2C to the VP16 activation domain. Activation of the MEF2C transcription activation domain by MyoD and Rb requires Ser387 of MEF2C, a site for phosphorylation by members of the p38 MAP kinase family. These findings fit a model in which MyoD and Rb stimulate p38 activity or, alternatively, inhibit MKP-1 activity, resulting in enhanced phosphorylation of MEF2C.

These studies provide interesting insights into the interdependent roles of Rb, MyoD and MEF2 in the control of myoblast proliferation and differentiation, and also raise other interesting questions. How do Rb and MyoD stimulate activity of MEF2C? As MEF2 factors are also active in proliferating cells, in which they have been shown to stimulate expression of immediate early genes like c-jun, their activity is clearly not restricted exclusively to postmitotic cells. Understanding how MEF2 is activated under these different conditions is an important question for the future.

Regulation of muscle fiber type by MEF2

Adult skeletal muscle fibers can be generally classified into fast and slow twitch fiber types on the basis of contractile and metabolic properties, and gene expression patterns. The fiber-type characteristics of adult skeletal muscle are determined by the pattern of motor nerve innervation. Tonic motor nerve stimulation results in slow fiber gene expression, whereas lack of stimulation evokes a fast fiber gene expression program. It is thought that intracellular calcium levels are responsible for regulating fiber type gene expression.

The diversity of fiber types is essential for accomplishing specialized tasks. However, the composition of fiber types in a muscle can be altered under certain physiological conditions or disease. The molecular mechanisms which direct fiber-type specification under normal as well as pathophysiological conditions remain largely unknown.

In light of the importance of sustained elevation in calcium concentrations for slow-fiber gene expression, Chin et al. [30•] tested whether the calcineurin-dependent protein phosphatase calcineurin might participate in slow-fiber gene expression. Calcineurin is localized to the cytoplasm and is activated by sustained low-amplitude
changes in intracellular calcium that result in dephosphory-
lization of nuclear factors of activated T cell proteins (NFATs), transcription factors that translocate to the nuclei-
us in response to dephosphorylation and act combinatorially with other factors to activate programs of gene expression. Intriguingly, several slow-fiber-specific skeletal muscle genes contain NFAT-binding sites adjacent to MEF2-binding sites in their control regions. Consistent with the potential involvement of calcineurin and NFAT signaling in the control of slow-fiber gene expression, activated cal-
cineurin activates several slow-fiber-specific promoters and the NFAT and MEF2-binding sites contribute to this response [30]. Moreover, treatment of rats with the cal-
cineurin inhibitor cyclosporin A results in an approximate twofold reduction in the number of slow skeletal fibers and a corresponding increase in fast fibers. Together, these results suggest that slow-fiber gene expression is dependent on calcineurin signaling and that the fast fiber program may represent a type of default program occurring in the absence of the positive signal for the slow fiber program.

The role of NFAT proteins in fiber-type-specific gene expression has recently been challenged. The slow-fiber-
specific enhancer from the troponin I slow gene, termed SIRE, was modified by replacing its native E-box with that from a fast fiber enhancer [31]. This chimeric con-
struct, which also lacks the NFAT-binding site, continued to express in a slow-fiber-specific manner in transgenic mice, indicating that NFAT and myogenin bHLH proteins are not essential to fiber-type specificity. Importantly, this chimeric construct retained intact MEF2, CACC, CAGG, and bisoid-like motifs.

Although the role of NFAT in slow-fiber gene expression remains in question, there is increasing evidence that MEF2 proteins, in concert with the calcineurin inhibitor calcineurin, are critical to the control of the slow-
fiber-specific gene expression program. The MLC2slow promoter is highly upregulated in slow fiber muscles. Mutational analysis of this promoter by direct injection of various MLC2slow reporter constructs into adult rodent muscle demonstrated that MEF2 and CACC cis-elements are required for fiber-type specificity, but E-box sequences are dispensable [32]. Whereas mutation of the MEF2 site resulted in a marked reduction of reporter activity in slow fibers, there was an increase in expression upon mutating the CACC element in fast fibers, but no substantial effect on reporter activity in slow muscle. Overexpressing MEF2 upregulated reporter activity in fast fibers only when the CACC motif was mutated, indicating that MEF2 is neces-
sary, but not sufficient, to confer fiber-specific expression. It was subsequently shown by gel shift analysis that endogenous MEF2 proteins bind the functional MEF2 sites of the MLC2slow promoter and that Sp1 is part of a MEF2 binding complex in slow muscle extracts.

Conclusions

Our understanding of muscle development has reached a point at which many (or even most) of the components of the transcriptional circuitry and signaling pathways responsible for specification and differentiation of myoblasts have been identified. It is clear that the partnership of MEF2 and MyoD lies at the heart of many aspects of muscle development. Although we are beginning to understand how the expression and activity of these factors are regu-
lated, many important questions remain. How, at a mechanistic level, does myogenic bHLH and MEF2 factors establish the code for skeletal muscle transcription? Do they uniquely recognize only those transcriptional control regions with a specific combination of cis-elements or do they form a special ternary complex that can specifically derepress the chromatin surrounding muscle-specific genes? How do the functions of the different MEF2 fac-
tors differ and what are the roles of different heterodimeric combinations of MEF2 isoforms? How does MEF2 regu-
late such diverse sets of genes in different cell types (e.g. cardiac, skeletal and smooth muscle, neurons and T cells) and what are the mechanisms that activate MEF2 factors in response to intracellular signaling?

Finally, a major issue for the future will be to understand muscle gene transcription at the whole genome level. Thus far, most studies of muscle transcription have involved a reductionist approach to focus on the specific DNA sequences and trans-acting factors that control indi-
vidual muscle-specific genes. With the rapid development of DNA array technology, it will soon be possible to view myogenesis in more global terms using expression patterns of large gene sets and to ultimately link our understanding of the mechanics of muscle transcription to a broader understanding of myogenesis at the genomic level.

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

• of special interest
•• of outstanding interest


This paper shows that MyoD mutants defective in muscle gene activation retain the ability to interact with MEF2. Thus, formation of a myogenic bHLH-MEF2 complex is insufficient to activate myogenesis. The results also demonstrate that the MyoD basic region is required for transcription of an activation signal in conjunction with MEF2. The essential role of the MyoD basic region for transcriptional activation can be bypassed by fusion of
This paper describes a novel mechanism for inhibition of MEF2 activity by TGF-β signaling. The results may explain how TGF-β blocks myogenesis, under certain conditions, with or without the Notch signalling pathway. The study also suggests that p21 CIP1 and p57 KIP2 act in parallel with Notch at an early step in the myogenic pathway.


Zhang P, Wang C, Lu D, Firestein M, Harper JW, Ellouzi Sf: p21(CIP1) and p21(CIP1)-like protein kinases, a role for c-Jun NH2-terminal activated protein kinase in the control of myoblast differentiation and shows that these double mutant mouse phenocopy myogenin mutant mice and contain myoblasts arrested in the differentiation step. The study also suggests that p21(CIP1) and p27(KIP1) are in parallel with myogenin at an early step in the myogenic pathway.


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