Chapter 2

The Myogenic Regulatory Factors: Critical Determinants of Muscle Identity in Development, Growth and Regeneration

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1. Introduction

The discovery of MyoD [1] was a landmark in our understanding of the processes leading to muscle cell differentiation. In this study a single cDNA was isolated which could induce conversion of fibroblasts into muscle cells [2]. This striking finding remains one of the clearest examples of a master regulator of cell fate and has made myogenesis an excellent paradigm for the understanding of how cell fate is induced and executed.

Other related genes were soon identified and three other closely related proteins have been isolated: Myf5 [3], Myogenin [4-6] and MRF-4 [7-9], which share the ability of MyoD to activate muscle gene expression. Together these are known as the Myogenic Regulatory Factors or MRFs. All of these genes are expressed during embryonic myogenesis exclusively in myogenic cells [10-14] although there are differences in the timing and stages of myogenesis, reflecting underlying differences in the roles of the MRFs in muscle cell commitment and differentiation [15, 16].

1.1. Muscle development

In vertebrate embryos muscle is derived from paraxial mesoderm which lies adjacent to the midline of the developing embryo [17]. In the head unsegmented mesoderm produces the branchial and extra-ocular muscles [18] while some of the neck muscles are derived from more lateral occipital mesoderm [19].

Grafting experiments in avian embryos, where quail mesoderm is grafted into chick embryo hosts, have demonstrated that all the muscles of the trunk are derived from somites, segmentally repeated epithelial structures that arise from the paraxial mesoderm [20, 21]. As

they differentiate somites produce the dermomyotome, a 'C' shaped epithelium containing proliferative muscle precusors (myoblasts) that express the transcription factor Pax3 [22].

Somites can be divided into two major domains: epaxial, located dorso-medially, and hypaxial, located ventrolaterally. Muscles arising from these domains correspond to the adult epaxial and hypaxial muscles which are innervated by the dorsal and ventral ramus of the spinal cord respectively. Cells from the dermomytome migrate around the edges of the dermomyotome to form an underlying layer, the primary myotome [23, 24], where the MRFs are first expressed and muscles begin to differentiate.

The muscles of the limb are also derived from somites but are generated when myoblasts delaminate from the hypaxial dermomyotome and migrate into the forming limb bud [17, 25]. This process is regulated by production of HGF/SF from the lateral mesoderm at limb levels which induces migration of myoblasts, to maintain them in a proliferative state and to delay MRF expression [26-28]. The expression of MyoD in these different muscle groups during embryo development is shown in figure 1.

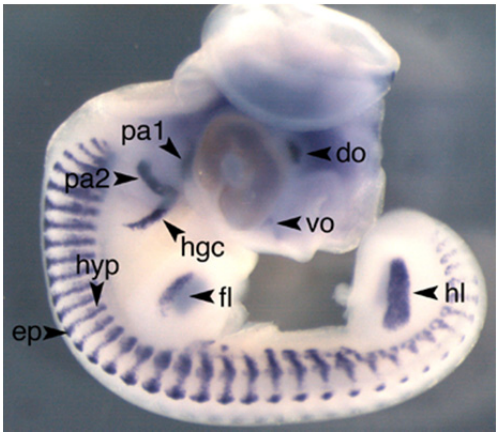

Figure 1. In situ hybridisation to show expression of MyoD in an HH stage 24 chicken embryo (approximately equivalent to mouse E11 or human Carnegie stage 16, around 40 days). Staining is seen in trunk muscle precursors in epaxial and hypaxial somites (ep, hyp), limb muscles of fore- and hindlimb buds (fl, hl), jaw and facial muscles in the pharyngeal arches (pa1, pa2), tongue muscle precursors in the hypoglossal chord (hgc) and the dorsal and ventral oblique extraocular muscles (do, vo)

Myogenesis in each of these different muscle groups, head, epaxial, hypaxial and limb, is regulated differently in the embryo [17]; however the MRFs play a key role in all of them and are part of a core transcriptional programme that operates in all skeletal muscles.

2. Regulation of the MRFs

Several signalling systems have been shown to affect MRF expression during development. It is notable that different sets of muscle precursors are regulated by separate sets of signals and, even with a single somite, there are distinct inductive pathways in hypaxial and epaxial

regions. In this section I will briefly review some of the molecular signals that have been shown to regulate MRF expression.

2.1. Signalling molecules regulating MRFs

2.1.1. Wnt

The signals induced by Wnts, the vertebrate homologues of the *Drosophila wingless* gene, are broadly divided into canonical and non-canonical types. Canonical signalling acts via β-catenin and the activation of TCF/LEF transcription factors [29] while non-canonical signalling acts via planar cell polarity or calcium dependant mechanisms [30] although these pathways are not always as clearly distinct as this division implies [31].

Explant culture of somites from chicken embryos demonstrated that signals from the neural tube and notochord are required for induction of MyoD [32] and Myf5 [33], an activity that can be recapitulated by the addition of purified Wnt-1 or Wnt-3 and low levels of Shh [34]. Mouse mesoderm explants exposed to Wnt-1 activate an epaxial, Myf5 dependant programme while exposure to Wnt-7a seems to induce a hypaxial, MyoD dependant myogenesis [35]. Wnt7 has also been implicated in the regulation of satellite cell activation via induction of MRF expression [36, 37].

In vivo Wnt1 and Wnt3a are secreted by the dorsal neural tube and are able to induce MyoD expression in the epaxial myotome, probably via β-catenin signalling [38]. In contrast, in limb muscles, Wnt-6, expressed in the limb ectoderm, has been shown to positively regulate Myf5 while downregulating MyoD [39]. In the limb induction of MRF expression is independent of β-catenin signalling although it is required for later myogenic differentiation [40].

2.1.2. Shh

The Sonic Hedgehog signalling pathway, which regulates the activity of the Gli family of transcription factors, is found in numerous inductive and patterning systems during development and plays a critical role in myogenesis [41, 42].

In somite explants induction of MyoD by Wnt is only observed in the presence of Shh [34]. This requirement for Shh signalling has been confirmed in vivo as loss of MyoD expression following notochord and floorplate removal can be rescued by grafting a Shh soaked bead into the excised region [43]. Analysis of mouse embryos lacking Shh shows this signal also controls expression of both Myf5 and MyoD in the epaxial somite [44].

Shh expression in the developing limb has been primarily analysed in its central role in patterning the anterior-posterior axis [45]. However, in contrast to its role as a positive inducer of MRF expression in epaxial somites, in limb muscles ectopic Shh expression delays MRF expression and maintains proliferative myoblasts, ultimately leading to muscle hypertrophy [46, 47].

2.1.3. BMPs

Bone morphogenetic proteins, members of the TGF-β family [48], are well characterised repressors of myogenic differentiation. BMP4 from the lateral mesoderm regulates formation of the hypaxial somite and represses MyoD expression [49]. In the epaxial myotome BMP signals must be inhibited for myogenesis to proceed and Wnt-1, from the dorsal neural tube, induces expression of noggin, an inhibitor of BMP signalling [50, 51]. The inhibitory effects of Shh in limb myogenesis are also mediated, at least in part, by induction of BMP expression [46].

2.1.4. Notch

Notch signalling can have either positive or negative effects on MRF expression, depending on context. Neural crest cells expressing the Notch ligand Delta migrate past the epaxial somite where they activate Notch in the myoblasts of the dorso-medial lip. This then induces expression of Myf5 and the beginning of myogenic differentiation [52]. In limb muscles Notch signalling does not affect Myf5 expression but does inhibit MyoD induction [53].

2.1.5. FGFs

In vertebrates there are 22 members of the fibroblast growth factor (FGF) family which act via four receptor tyrosine kinases, the FGF receptors [54]. Grafting of FGF4 or FGF8 beads adjacent to somites leads to the loss of expression of MyoD and other myogenic markers [55, 56] but induces the expression of the tendon marker scleraxis. However later in somite development FGF from the myotome induces epithelial to mesenchymal transition and translocation of dermomytomal cells into the central region of the myotome [57], a process known to contribute to the satellite cells of the adult [58]. In limbs FGF4 beads have been reported to downregulate MyoD expression [59] although the receptor through which it is though to signal, FGFR4, is required for limb muscle cell differentiation [60] as expression of a dominant negative form of the receptor leads to decreased MyoD expression.

As is often the case in development the response to signalling events is context dependent and it is becoming clear that there are many variant myogenic programmes which are activated in different muscle groups; uncovering these distinct regulatory mechanisms remains an exciting area of muscle biology.

2.2. Molecular and genomic regulation

The ability of the MRFs to induce muscle specific gene expression means that they, in turn, are tightly regulated as inappropriate expression of MRFs could lead to production of ectopic muscles.

To determine the genomic elements controlling the highly specific expression of Myf5 a series of mice have been generated where reporters, such as LacZ, are expressed under the control of specific regions of the surrounding genome. These have revealed a system of remarkable

complexity where Myf5 is controlled by a combination of promoter and enhancer elements that span 150kb of chromosome. The overall expression pattern of Myf5 is made up of numerous smaller patterns, each with a specific enhancer driving Myf5 expression in a particular subset of muscle precursor cells [61-66]. One particularly striking example of the convergence of mouse genetics and experimental approaches is the finding that that in one of these regions, the early epaxial enhancer, binding sites for both Lef and Gli have been identified [67]; these are the molecules responsible for transducing Wnt and Shh signals that had been previously implicated in MRF induction in somite explant experiments [34, 35]

One intriguing exception to the muscle specific expression of Myf5 is found in some regions of the mouse CNS [68]. The other MRFs are not expressed here and, as there is no muscle present, the role of this neural expression was unclear. More recently it has become clear that a genomic reorganisation in evolution is responsible for this inappropriate expression. However to prevent the activation of muscle specific genes in the nervous system the mRNA transcribed here is not translated and endogenous microRNAs are able to repress the production of Myf5 protein [69].

Together this provides both positive and negative mechanisms for the regulation of Myf5. Once Myf5 is expressed it can then induce expression of the other MRFs which also regulate each other. The exception to this is Myf5 which is not induced either by itself or the other MRFs [70-72]. Because of this the other MRFs do not seem to require such complex regulatory regions and have rather simpler genomic control mechanisms.

MyoD expression is largely regulated by two enhancers, the core enhancer located at -20kb, and the distal regulatory region (DRR) loicated at -4kb. These have been extensively analysed by generating enhancer reporter fusions and mutational analysis in mice [73-79] and birds [80] which have shown that the core enhancer is required for the onset of MyoD expression while the DRR has a more important role in later differentiation. Several factors have been indentified which are required for MyoD transcription including Pax3 which acts in concert with DNMRT and Myf5 [81] Six1 and Six4 [82, 83], Pitx2 [84], Sim2 [85] and Foxo3 [86]. Although Myf5 can activate MyoD it is not required in all cases and MyoD can be induced independently by this array of transcription factors [87].

Myogenin expression can be largely recapitulated with a reporter containing 4kb of upstream sequence [11]. Myogenin expression is regulated, at least in part, by MyoD along other factors, such as NFAT [88], which recruit chromatin remodelling complexes to the myogenin locus [89, 90].

3. Biochemical activity of the MRFs

The MRFs are basic-helix-loop-helix (bHLH) proteins, members of a widespread family of transcription factors found throughout eukaryotes [91]. An alignment of the protein sequences of the four MRFs is shown in Figure 2 with important functional domains highlighted. bHLH proteins are well characterised regulators of differentiation and have been implicated in many developmental systems including ear [92], cardiac [93] and neural

differentiation [94]. bHLH proteins bind specific DNA motifs, known as E boxes, normally as heterodimers in combination with the ubiquitously expressed E12 and E57 proteins [95].

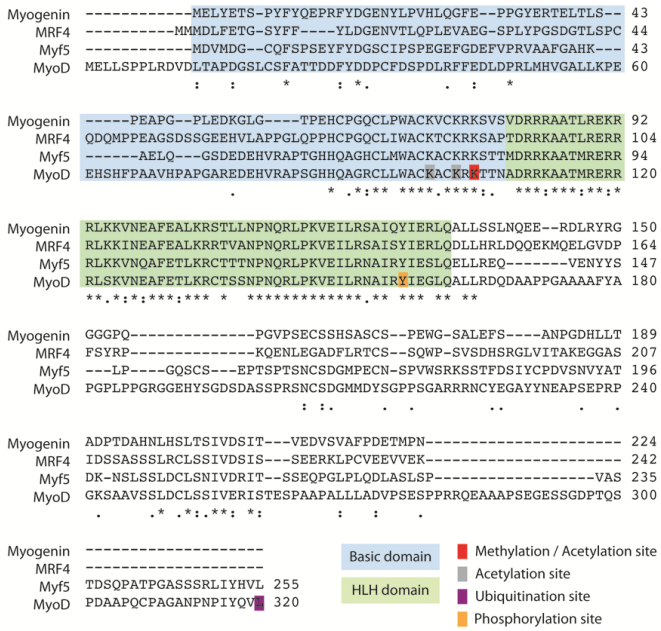

Figure 2. Clustal alignment of human MRF sequences. Basic domain is highlighted in blue, helix-loop-helix domain in green. MyoD methylation / acetylation sites shown in red, acetylation sites in grey, ubiqutination site in purple and phosphorylation site in orange.

Although they have similar biochemical activities in vitro and can bind E boxes in DNA it is clear that there are distinct biochemical activities and functions for the individual MRFs. MyoD and myogenin have been directly compared in their ability to bind to and activate transcription from several muscle specific promoters, such as the chicken myosin light chain, [96, 97] as well as in more global genome binding analysis [98]; it is clear that they bind distinct subsets of promoters and have different sets of target genes. Similar experiments have shown different DNA binding activity of MRF4, MyoD and Mgn [99] while comparison of Myf5 and MyoD activity has mapped part of this differential transcriptional activity to the N and C terminal regions of MyoD which co-operate to give increased transcriptional activation of specific genes which are not activated by Myf5 [100].

An interesting question is how MRF binding to DNA is able to specifically activate muscle gene expression. Recruitment of MyoD to E boxes can be enhanced by the presence of DNA quadruplex structures in promoters [101]; however E boxes are widespread throughout the genome and global analysis of MyoD binding suggests it is able to interact with a large number of these even though they are not associated with muscle specific genes and so do not result in transcriptional activation [102]. Part of the answer to this is that while MRF binding is required for muscle gene expression it is not sufficient and other transcriptional

activators, such as the Six [82, 83] and Pbx proteins [103, 104] are also required at muscle gene promoters to drive expression. However the widespread binding of MyoD may have a broader function and it has been suggested that this can lead to generalised remodelling of the genome in preparation for myogenic differentiation [102]. A similar role has been proposed where MyoD binding is first required at distal enhancers of repressed myogenic genes which have promoter elements inaccesible to transcription factor binding due to their chromatin structure. Interactions between these distal enhancers and more proximal promoters leads to chromatin remodelling at that locus. This opens the promoter and makes it available for MRF binding [105]. It is tempting to speculate that this may be the reason for the pulse of Myf5 expression in paraxial mesoderm prior to somite formation and that this is preparing cells for subsequent inductive events and thus enabling myogenesis.

A recent comparison has also shed light on the specificity of target gene activation by MyoD. Comparison of MyoD binding with a neuronal bHLH protein, NeuroD2, has identified both common and specific E box sequences that these proteins can bind. MyoD specific E boxes are linked to transcription of muscle specific genes while binding to the common E boxes results in broader epigenetic modifications [106].

The activity of MyoD is also regulated by several biochemical modifications and interactions. MyoD is regulated by ubiquitination at its N terminal which targets it for degradation [107, 108]. MyoD is also negatively regulated by methylation which impairs its ability to induce differentiation [109]. MyoD is also acetylated [110] and phosphorylated [111], with both events seeming to enhance MyoD activity. Many of the residues modified in MyoD are conserved across the other MRFs (see figure 2) and it is possible that they are also regulated in this way.

As well as interaction with the E proteins required for transcriptional activity MyoD has also been reported to interact with a wide range of other proteins including c-jun [112], CTCF [113], BAF60c [114], CLP-1 and HDAC at the cyclin D promoter [115], TAZ at the Mgn promoter [116] and β-catenin [117]. MyoD can also interact with cell cycle regulators such as pRB [118] and cdk4 [119] to induce cell cycle withdrawal directly during myogenic differentiation.

This range of interactions shows clearly that the control of MyoD activity is a carefully regulated process and subject to numerous levels of control.

3.1. Targets of MRFs

The biochemical differences in the MRFs contribute directly to their distinct functional roles. Myf5 is able to activate genes required for myogenic commitment while MyoD can also switch on differentiation genes [100]. Similarly MyoD and MRF4 have distinct sets of targets and differentially affect proliferation and differentiation [120].

Myogenin acts downstream of MyoD and is often only able to activate transcription from promoters which have already been bound by MyoD [98]. Myf5, MyoD and Myogenin binding of target sequences is also temporally regulated, providing another mechanism for specificity of target gene activation [121].

One of the best characterised MyoD targets is myogenin. MyoD can bind the myogenin promoter along with Mef2 (another transcription factor and MyoD target gene) [122, 123]. Myogenin, MyoD and Mef2 then co-operate with other transcriptional regulators, such as Six proteins, to activate muscle specific genes such as muscle myosins [96] or muscle specific microRNAs [71, 124] via demethylation of promoter elements [125].

4. Animal models of MRF function

Probably the most widely used animal models to study MRF function are transgenic mice. However knockout animals have shown surprisingly mild effects and mice lacking MyoD [126], Myf5 [127] and MRF4 [128, 129] are all able to develop apparently normal muscle although delays in myogenesis do occur in the limbs of MyoD [130] and somites of Myf5 [127] mutant animals. In contrast mice lacking myogenin have severe muscle defects and die soon after birth [131, 132].

Double knockouts of MyoD and Myf5 were originally reported to lack muscle [133] although subsequently it appears that these mice also lacked MRF4 expression as the targeting of Myf5 had also affected the closely linked MRF4 locus. In MyoD / Myf5 null animals which retain functional MRF4 this gene is able to compensate and initiate myogenesis [134]. Knockout mice have shown that the relationship between the different MRFs is complex and one probable explanation for the functional redundancy of these proteins is that in the absence of one another will be upregulated to substitute for it [126, 128]. The exception to this is myogenin which has a unique, non-redundant function [135] which cannot be compensated for by the other MRFs. Overlapping roles for MRFs are also demonstrated in mice lacking MyoD and MRF4 which have severe muscle defects [136]. It is also apparent that Myf5 alone is not sufficient to support myogenic differentiation as in mice lacking the other MRFs myogenesis is initiated but not maintained [137]. This specificity of individual MRF function has also been demonstrated in other animal models such as Xenopus [138], zebrafish [139] and chickens [70, 71] although it is striking that some specific functions of MRFs have changed during evolution. An example of this is the regulation of the muscle specific microRNA miR-206 which appears to have different requirements for MRF expression in mice, chickens and fish [70, 71, 140].

The role of Myf5 has been further examined by the production of transgenic mice which express diptheria toxin under the control of Myf5, thus ablating all Myf5 expressing cells in the embryo. Fascinatingly these mice develop morphologically normal muscle [141, 142], suggesting that a Myf5 independent population of myoblasts are present and can expand to fill the niche left by loss of Myf5 expressing cells. This correlates well with data showing that distinct regulation of MyoD and Myf5 defines different subsets of cells based on reporter gene expression [16].

5. Summary

Although great strides have been made in understanding the MRFs at biochemical, genomic and whole animal levels there remain significant unanswered questions. Among these is

issue of what are target genes of each MRF in vivo and how do they differ in their activity in different muscle types. Understanding the answers to these questions will provide key insights which will directly influence both basic science and regenerative medicine.

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