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Skeletal muscle programming and re-programming

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Abstract

The discovery of the transcription factor MyoD and its ability to induce muscle differentiation was the first demonstration of genetically programmed cell transdifferentiation. MyoD functions by activating a feed-forward circuit to regulate muscle gene expression. This requires binding to specific E-boxes throughout the genome, followed by recruitment of chromatin modifying complexes and transcription machinery. MyoD binding can be modified by both cooperative factors and inhibitors, including microRNAs that may serve as important developmental switches. Recent studies indicate that epigenetic regulation of MyoD binding sites is another important mechanism for controlling MyoD activity, which may ultimately limit its ability to induce transdifferentiation to cells with permissive epigenetic ‘landscapes.’

MyoD: a muscle transdifferentiation factor

Early studies in myogenesis and other programs of cell differentiation led to the hypothesis that complex programs of differentiation were controlled by a small number of master regulatory, or master switch, genes [1-3]. Myogenesis represented an ideal model system for identifying a master regulator [4, 5], and led to the identification of MyoD, a single cDNA that was sufficient to convert fibroblasts to skeletal muscle cells [6, 7]**. Subsequently, MyoD was shown to convert a number of cell types into muscle, including dermal fibroblasts, chondrocytes, smooth muscle, retinal pigmented epithelial cells, adipocytes, and melanoma, neuroblastoma, osteosarcoma, and hepatoma cells [8, 9]. These studies provided the first demonstration of genetically programmed cell transdifferentiation.

Muscle differentiation requires the coordinated expression and silencing of muscle and non-muscle genes, respectively. Over the past 2 decades, there has been significant progress in our understanding of how MyoD regulates this gene program [10]. Early work revealed that MyoD functions by binding to DNA as a heterodimer with widely expressed E-proteins in order to activate gene transcription [11]. Heterodimer binding is specific for the sequence CANNTG, termed an E-box. Both DNA binding and dimerization are mediated through a

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conserved basic-helix-loop-helix (bHLH) domain. Over 100 bHLH domain containing proteins have been identified in humans [12], including four related myogenic bHLH proteins: MyoD, MYF5, Myogenin, and MRF4. In this review, we will focus on MyoD as the most intensively studied member of these myogenic bHLH proteins.

Mechanisms of muscle gene regulation by MyoD

MyoD requires a minimum of two E-boxes, or an E-box and a site for a cooperative co-activator, for activation of reporters *in vitro*. Cooperative binding between the two E-box sites leads to enhanced affinity as demonstrated by dissociation assays [13]. Subsequent to binding, the recruitment of histone acetyltransferases (HATs) and chromatin remodeling complexes leads to histone acetylation and chromatin opening [14]. MyoD has recently been shown to also interact directly with the Baf60c subunit of the SWI/SNF chromatin-remodeling complex [15]*. Thus, MyoD binding creates a transcriptionally permissive local chromatin environment. MyoD can be recruited to inaccessible E-box sites through interaction with PBX [16], and perhaps other factors. Within the myogenin promoter, for example, PBX is bound in myoblasts prior to differentiation, and recruits MyoD during differentiation. The subsequent recruitment of chromatin remodeling complexes and histone acetyltransferases makes the regional E-box binding sites more accessible to MyoD.

Earlier work also showed that DNA binding could occur without transcriptional activation. Positive control mutations (i.e., mutations that prevent transcriptional activation by MyoD but do not alter DNA binding at E-box sequences) within the bHLH domain revealed the presence of a myogenic code within the basic region that is required for muscle specific gene activation [17]**. Specifically, replacement of the MyoD basic region with that of the E12 E-protein leads to loss of myogenic conversion, and back mutation of 3 residues to the sequence 'ATK' restores this activity. Most remarkable, however, was that E12 mutants with these three MyoD residues activated muscle gene expression in fibroblasts, albeit much less efficiently than MyoD [17]. It is interesting that these residues are also important for cooperative binding of MyoD at its E-box binding site [18] and for recruitment of other co-activators [19].

While many of the early studies were limited to evaluation of individual MyoD binding sites, recent genome-wide expression and binding assays have provided a broader picture of the MyoD network. Expression arrays and MyoD chromatin immunoprecipitation (ChIP) analyses revealed that MyoD directly bound to the regulatory sequences of genes activated both early and late in the program of muscle gene expression, achieving temporal regulation through a feed-forward circuit [20, 21]. At the same time MyoD is activating muscle gene expression, it is suppressing the expression of other non-muscle genes through a negative feed-forward circuit, termed an incoherent feed-forward loop. MyoD activates the expression of the zinc-finger proteins ZNF238 and ZEB, which in turn can bind to E-boxes of genes regulated by MyoD and displace or prevent MyoD binding and activity [22]*[23]*. Another zinc-finger protein SNAIL also competes for MyoD binding to E-boxes and is targeted by mir-206 [24]*, a MyoD induced micro-RNA that also suppresses other genes during muscle differentiation [25]. Mir-206, in addition to downregulating *Snail2*, also integrates multiple muscle differentiation signals in pediatric skeletal muscle tumors, and can lead to their differentiation when ectopically expressed [26].

Although MyoD binds to thousands of sites in muscle cells that are associated with genes activated by MyoD during differentiation [22, 24], the majority of MyoD binding sites are not associated with regional gene transcription [22, 27]*. While the function of these latter binding events remains to be completely elucidated, this genome-wide MyoD binding is associated with significant regional histone acetylation, which suggests a role for MyoD as a

factor that alters the epigenetic landscape by recruiting HATs to regions throughout the genome, perhaps through interactions with 'beacon' factors like PBX. This is consistent with the observation that transdifferentiation by MyoD is limited by cellular context, suggesting either that MyoD cannot act as a 'pioneer' factor [28], or that its role as a pioneer is insufficient for muscle differentiation in certain cell types. In support of the former, work from our group suggests that lineage-determined epigenetic context determines the set of genes transcriptionally activated by MyoD or by the related neurogenic bHLH transcription factor NeuroD2 [27]. When ectopically expressed in the P19 mouse embryonal carcinoma cell line, MyoD has limited ability to induce muscle differentiation [29]. Examination of the MyoD-binding and chromatin accessibility profiles within P19 cells indicates significant differences with fibroblasts, although the sequence of MyoD targeted E-boxes is identical, with strong preference for E-boxes containing CC or GC in the central two positions. MyoD binding in P19 and other cells correlates strongly with the nuclease accessibility assessed prior to the introduction of MyoD. Thus, part of the MyoD-mediated myogenic program is dictated by the internal nucleotide sequences of the MyoD E-box motif, which differs from that of the neurogenic bHLH factor NeuroD2, while another part is dictated by the epigenetic context that determines the set of E-boxes available for MyoD binding, either directly or through interaction with PBX-like factors that might have a combinatorial expression pattern that establishes competence in different cells. This model is consistent with recent genome wide studies that show an interdependence of chromatin accessibility and transcription factor binding. For example, studies with the glucocorticoid receptor (GR) demonstrate that accessibility of binding sites precedes and predicts GR binding [30]**, and that pre-existing AP1 binding establishes the chromatin accessibility at GR-binding sites and facilitates GR recruitment [31].

Epigenetic regulation of muscle differentiation is in part controlled by the Polycomb group (PcG) proteins, a family of epigenetic regulators involved in controlling developmental fates, establishing lineage identities, and maintaining stem cell states, all through the repression of specific genes [32]. Conditional knockout of EZH2, a component of the Polycomb repressive complex 2 (PRC2), in muscle satellite cells results in aberrant expression of neuronal, retinal, cardiac, and chondrocyte genes [33]*, and abnormal differentiation of these cells. The up-regulation of alternative lineage genes was associated with loss of repressive H3K27 methylation and PolII recruitment to the abnormally accessible chromatin. Intriguingly, EZH2 loss did not have a similar effect on MyoD-expressing committed muscle cells. It remains unknown, however, whether the absence of EZH2 can affect the landscape of MyoD binding.

Together, these studies suggest that the ability of MyoD to activate the muscle gene program is limited by the epigenetic determination of the binding sites available for MyoD. Thus, lineage-specifying factors might establish different chromatin 'landscapes' that determine the available set of E-boxes and whether MyoD will bind and activate a coherent myogenic feed-forward circuit or activate an incoherent set of genes. While the molecular mechanisms that establish these chromatin landscapes are not known, the combinatorial expression of homeodomain factors and lineage specifying factors might play a role.

Our recent finding on the cell-type restriction of myogenic conversion by MyoD [27] ultimately needs to be reconciled with our earlier studies showing that MyoD can convert cells of all three embryonic cell lineages to skeletal muscle[8]. The earlier study used cell lines that might have compromised some aspects of their lineage identity with passage in cell culture, comparable to an epithelial-to-mesenchymal transition (EMT) that can occur in cell culture, and it will be of interest to revisit and rigorously test the lineage restriction of MyoD-conversion in *in vivo* expression studies. Indeed, *in vivo* studies in *C. elegans* show that developmental lineage specification is associated with a restricted ability to be

converted to muscle by MyoD [34], and that this developmental loss of plasticity is regulated, at least in part, by chromatin repression through the mes-2/E(Z) polycomb repressive complex (PRC) protein [35]. Similarly, loss of PRC2 in *C. elegans* germline cells made them capable of being converted to muscle by ectopic expression of MyoD, as well as conferring the ability to convert to neurons [36]. Expression of MyoD in *X. laevis* showed that broad activation of its target genes was restricted to the mesodermal ring around the blastopore [37], and ectopic expression of NeuroD family members in *X. laevis* showed ectopic neurogenesis that was relatively limited to the epidermal cells [38, 39]. These *in vivo* indications that lineage specification restricts the ability of cells to respond to MyoD is consistent with our recent demonstration that lineage-specific chromatin accessibility to binding sites determines the transcriptional program activated by MyoD and suggests that conversion to a mesenchymal lineage might greatly potentiate the ability of MyoD to induce the program of skeletal muscle differentiation. Ultimately, our ability to reprogram multiple cell types into skeletal muscle may be enhanced if we can alter the chromatin landscape to make critical MyoD binding sites available, possibly through a mechanism similar to EMT.

Clinical application of muscle transdifferentiation technology

Since the discovery of MyoD and its transdifferentiation properties, Yamanaka's group has demonstrated that a set of 4 transcription factors can drive a somatic cell back to a pluripotent state [40]. More recently, other groups have used different combinations of transcription factors to directly convert cells from one type to another, including fibroblasts to neurons [41]*, pancreatic exocrine cells to insulin-secreting islet cells [42], and fibroblasts to cardiac myocytes [43-45]. In each of these cases, conversion was directed by combinations of transcription factors, with sustained high levels of these factors thought to be required for conversion of a relatively small number of cells [46]*. Evidence for direct conversion, rather than de-differentiation to a stem-like state followed by re-differentiation, includes: (1) lack of expression of typical stem or progenitor cell markers; (2) differentiation in a relatively short time frame (<1 week); (3) absence of significant cell division after introduction of factors. As with MyoD, however, the transdifferentiation mediated by these cocktails of transcription factors is limited to specific cell types. This again may be due to the restrictive epigenetic and cellular contexts of a cell at the time of exposure to these factors.

Replacement of damaged muscle tissue with healthy muscle cells represents a potentially curative strategy for a number of degenerative muscle diseases, including Duchenne muscular dystrophy. While early efforts were impeded by low rates of muscle conversion and difficulty isolating dedicated muscle precursors, studies have shown that direct conversion of fibroblasts to muscle cells by MyoD transduction can support muscle regeneration [47]. In addition, Darabi et al have been able to convert iPS cells to muscle by expressing PAX-3 or PAX-7 in embryonic stem cells followed by isolation of PDGF R+/Flk1- cells prior to transplantation [48]**. These muscle precursor cells were able to effectively engraft into injured host mouse muscle and resulted in improved function. These cells are also able to effectively populate the satellite cell compartment [49, 50], with potential for providing long-term regenerative capabilities. Importantly, isolation of these precursor cells prevented the generation of teratomas at the site of injection that result from engraftment of non-committed ES cells. Differentiation of ES cells by ectopic expression of MyoD has also been recently demonstrated at 90% conversion efficiency [51], representing another potential strategy for generating muscle cells. However, it remains to be seen if these cells can be transplanted successfully and function within the satellite cell compartment.

Conclusion

The ability of MyoD to reprogram cells to skeletal muscle was a landmark event in transdifferentiation. More than 25 years later we are still learning about the molecular mechanisms of lineage-determined cell fate restriction and the interplay between the chromatin landscape and the transcription factor networks. A better understanding of the molecular mechanisms that establish the epigenetic landscapes that permit or constrain the activity of differentiation factors like MyoD will likely give significant insight into the fundamental processes of transdifferentiation.

The recent advances in our ability to manipulate cell fate decisions promises to vastly increase our understanding of fundamental processes in developmental biology in the near future. A number of clear and achievable directions for scientific investigation lay before us, and may ultimately provide significant breakthroughs to assist with treatment of various human diseases, from replacement of injured or degenerated tissues to curing cancer.

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Figure 1.

Transcription factor binding specificity and chromatin context work together to control cell differentiation. (a) Schematic nucleus showing only a few chromatids and a representation of MYOD E-boxes associated with muscle genes (red) or not associated with muscle genes (blue). (b) Chromatin context appears to be pre-determined by the cell lineage (open, accessible compartments shown in white; closed, inaccessible compartments in gray). An outstanding question is how such chromatin contexts are determined in the first place, or modified, by the different cell lineages. (c) Exogenous expression of MYOD converts MEFs into muscle, but not P19 mouse embryonal carcinoma cells. The developmental outcome is a consequence of the interplay between MYOD binding patterns and the pre-set chromatin context. Photomicrographs at the bottom show muscle cells derived from differentiated MEFs appearing on the left and undifferentiated P19 cells on the right (nuclei in blue are DAPI stained; muscle cells in green due to staining with myosin heavy chain antibody; colors merged in both photomicrographs)