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MOLECULAR MECHANISMS REGULATING MYOGENIC DETERMINATION AND DIFFERENTIATION

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1. ABSTRACT

The myogenic regulatory factors are necessary for the determination and terminal differentiation of skeletal muscle. Gene targeting experiments have demonstrated that MyoD and Myf5 are important for myogenic determination whereas myogenin and MRF4 are important for terminal differentiation and lineage maintenance. During development, all trunk skeletal muscle is derived from the somite. Two spatially distinct sources of myogenic progenitors are defined by the expression of *MyoD* or *Myf5* and these give rise to hypaxial and epaxial musculature. Both *in vivo* and *in vitro* analyses have provided a detailed picture regarding the molecular events controlling lineage determination, cell migration, terminal differentiation and tissue repair. Signal transduction pathways regulating cell cycle, protein-protein interactions and myogenic factor gene activation are implicated in the regulation of myogenesis. Recent experiments examining the origin and stem-cell capacity of satellite cells suggest that these cells may originate from the vascular system, are multipotential and may be useful for the treatment of several degenerative diseases.

2. INTRODUCTION

Skeletal muscle represents an ideal model system for the study of many biological problems. Distinct molecular markers exist that permit detailed analyses of myogenic determination, myoblast proliferation and terminal differentiation. The myogenic regulatory factors are vital to the determination and maintenance of skeletal muscle. During development, the induction of *MyoD* and *Myf5* expression defines the origin of myogenic progenitor cells (mpcs) that are responsible for forming distinct muscle groups of the adult organism. Gene targeting and transgenic mice have provided insight into the genetic relationships within the myogenic regulatory factor family and with molecules expressed within presumptive myogenic lineages. Interesting new insights have been uncovered explaining the molecular mechanisms that govern both proliferation and terminal differentiation. A number of signaling pathways have been shown to regulate myogenesis during development and regeneration of damaged tissue in the adult. These pathways regulate cell cycle progression, protein-protein interactions and transcriptional activity of the myogenic factors.

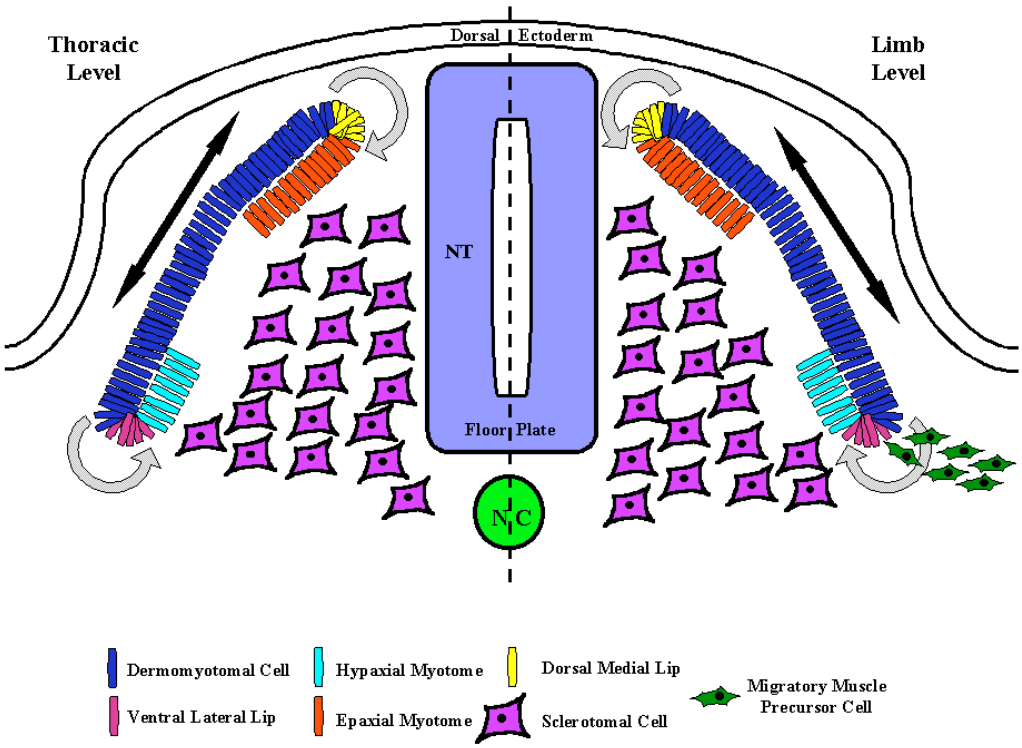

Figure 1: Somitic origin of the trunk musculature. The left side depicts events that occur in somites at the thoracic level and the right side shows events at limb-level somites. Dermomyotomal expansion leads to the extension of cells from the dorsomedial lip (DML) to a position beneath the dermomyotome. This marks the formation of the epaxial myotome which can be identified by *Myf5* expression. A similar extention occurs at the ventrolateral lip (VLL) forming the hypaxial myotome. The cells of the hypaxial myotome predominantly express *MyoD*. At the limb level, cells in the VLL delaminate and migrate to the developing limbs. These cells are *Pax3*, *Lbx1*, *c-Met* and *Msx1* positive. Upon arrival, these cells down-regulate *Pax3* and initiate expression of the myogenic regulatory factors, in particular *MyoD*. Formation of the body wall musculature occurs via a continued ventral expansion of the myotome. It should be noted that the first appearance of the epaxial myotome occurs at day 8.5 whereas the first appearance of the hypaxial myotome is at day 9.5. NT=neural tube; NC=notocord.

Furthermore, the use of the *mdx* mouse, which represents a model system for Duschene's muscular dystrophy, is expanding our knowledge of skeletal muscle diseases and uncovering novel insights regarding satellite cell origins and potential therapies to alleviate the debilitating effects of these diseases.

In this review, we will provide the reader with a basic understanding of the molecular events that are responsible for regulating myogenic commitment, myoblast proliferation and terminal differentiation. Furthermore, we briefly outline exciting new developments in myogenic stem cell research.

3. DETERMINATION AND DEVELOPMENT OF THE MYOGENIC LINEAGE

3.1. Somitogenesis: formation of epaxial versus hypaxial musculature

The formation of somite pairs on either side of the neural tube marks a crucial event during vertebrate development (for review see 1). Somites form in a rostral to caudal direction and epithelization begins about day 7.5 postcoitum (p.c.) in the mouse. As development proceeds, somites become subdivided into the ventral sclerotome and dorsal dermomyotomal domains. Sclerotomal cells give rise to the vertebrae and the ribs whereas the dermomyotome gives rise to the dermis of the back and the adult skeletal musculature of the trunk (reviewed in 2 and 3). Early experimentation using quail-chick somite grafts demonstrated that medial and lateral portions of somite are patterned by secreted factors from surrounding tissues and give rise to two distinct populations of myoblasts (4,5,6). Cells of the medial portion of the somite give rise to the muscles of the deep back, or epaxial muscles, whereas the lateral portion develops into the muscles of the body wall and limbs, or hypaxial muscles (5).

In birds, myotome formation occurs in sequential stages (for review see 7 and 8). First, cells in the dorsomedial lip (DML) extend beneath the dermomyotome, exit the cell cycle, elongate and terminally differentiate (9,10). These pioneer cells mark the first appearance of the

myotome and are followed shortly after formation by a second wave of cells migrating from the rostral and caudal portions of the somite (11). The cells of the second wave originate in the DML and are dependent upon migration to enter the myotome from the correct position (11,12). Myotomal development from cells originating in the DML represents the epaxial portion of the myotome. A similar series of events occurs at the ventral lateral lip (VLL) of the dermomyotome leading to the formation of the non-migratory hypaxial portion of the myotome (12,13). Subsequent expansion of the myotome occurs from the more superficial to deep regions of the myotome (12).

At the limb level, events at the VLL occur differently (Figure 1). Cells of the VLL undergo an epithelial to mesenchymal transition, delaminate from the dermomyotome and migrate to regions of presumptive muscle development in the limbs (for review see 14). Limb muscle formation occurs in temporally distinct waves involving at least two populations of cells that give rise to primary and secondary myotubes (6). *In vitro* characterization has shown that these two cell populations are distinguishable on the basis of clonal morphology and media requirements (15,16,17). Moreover, the myosin heavy chain isoforms expressed by early and late cells differ suggesting primary myoblasts are destined to give rise to slow muscle fibers whereas secondary myoblasts give rise to fast muscle fibers (for review see 18). *In vivo* analyses of somite and limb grafts suggest that these early and late populations do indeed give rise to slow and fast fibers, respectively (19). Although injection of embryonic myoblasts into limbs of developing birds suggested that their lineage is maintained (20,21), experiments in adults support a model in which environmental cues, such as innervation, play a substantial role in determining fiber-type potential (22,23).

A third wave of migration, which represents the adult satellite cells, can be detected during the midfetal gestational stage in birds (24). These cells appear to be of somitic origin (25), and are responsible for the majority of postnatal skeletal muscle growth (26,27). *In vitro*, these cells can be phenotypically distinguished from primary and secondary myoblast populations (28,29,30). Interestingly, analysis of clonal cultures from adult avian muscle suggests that satellite cells express a phenotype that is consistent with their fiber-type origin, although continued passaging of these cells indicates phenotypic plasticity (29). Taken together, development of vertebrate trunk musculature involves multiple cell lineages that arise from spatially distinct regions of the somite. The myogenic regulatory factors (MRFs) are critical for the appropriate determination, development and maintenance of these skeletal muscle lineages. We will now discuss the current knowledge regarding the MRFs and how their expression and activity during embryogenesis is regulated.

3.2. The myogenic regulatory factors

The original cloning of MyoD and demonstration that it represents a master regulatory gene for the determination of skeletal muscle, ushered in a new era of research in skeletal myogenesis (31). This discovery lead to the cloning of three other factors namely Myf5 (32), myogenin (33,34), and MRF4/Myf-6/Herculin (35,36,37). In all cases, overexpresison of these factors converts non-muscle cells to the myogenic lineage, demonstrating their role in myogenic lineage determination and differentiation. Furthermore, the ability of each factor to initiate the expression of one or more of the other three suggests they form a cross-regulatory loop (38).

The MRFs belong to the basic helix-loop-helix (bHLH) superfamily of transcription factors which includes c-myc. The HLH domain is responsible for the dimerization of these factors with the ubiquitously expressed E-proteins, such as E12, E47, HEB, and ITF, and the basic domain is responsible for DNA binding (39,40,41). Heterodimers bind to the consensus E-box (CANNTG) DNA sequence motif found in the promoters of many muscle specific genes (40,42,43). The bHLH domains of the MRFs are highly homologous while the amino and carboxyl terminals show limited homology. Structurally, the MRFs contain several functionally distinct domains responsible for transcriptional activation, chromatin remodeling, DNA binding, nuclear localization and heterodimerization (44,45,46,47,48).

3.2.1. Developmental expression of the myogenic regulatory factors

During development the MRFs are expressed in a highly regulated spatial and temporal fashion (reviewed in 49,50). *In situ* hybridization analyses demonstrate that MRF expression occurs in slightly different patterns in epaxial versus hypaxial muscle. *Myf5* expression is detected in the dorsomedial portion of the somite at day 8 p.c. and at day 9.5 in the lateral, or hypaxial domain of the somite (51,52). *Myogenin* is first detected at day 8.5 p.c. and remains detectable throughout fetal development (53). *MRF4* expression is detected transiently between days 10 and 11 and then reexpressed from day 16 onward to become the predominant MRF expressed in adult muscle (54,55,56). *MyoD* expression is first detected approximately day 9.75 in the hypaxial somitic domain and continues to be expressed throughout development (54,57). In the limb bud, the temporal appearance of these factors is slightly different. Although *Myf5* expression is again detected first, it is followed very quickly by *MyoD* and *myogenin* which are detected from day 10.5 onward (51,53). Unlike observations in the somite, *MRF4* is not transiently expressed during limb development but is first detected at day 16 and becomes the predominant MRF expressed in the adult (54,55). Analysis of protein expression has confirmed the in situ hybridization results with the dorsal and ventral subdomains of the myotome predominantly expressing Myf5 and MyoD, respectively (50).

3.2.2. Lessons from gene targeting

Targeted inactivation of the MRFs has provided a great deal of insight into the nature of lineage determination, lineage maintenance and their genetic relationships. Mice lacking a functional copy of *MyoD* are viable without any obvious defects in skeletal muscle (58). In a similar fashion, targeted inactivation of the *Myf5* gene

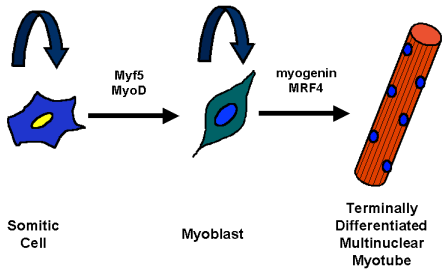

Figure 2: Functional and genetic relationships of the MRFs. Gene targeting experiments indicate that Myf5 and MyoD are required for the determination of the myogenic lineage. By contrast, terminal differentiation is dependent upon myogenin and MRF4.

gives rise to mice with seemingly normal muscle although these mice die perinatally due to a severe rib development defect (59). *Myf5* null mice do not show changes in the expression pattern of the other MRFs but do demonstrate a delay in myotome development (59). Mice lacking both *MyoD* and *Myf5* genes show a complete absence of myoblasts and muscle fibers. This demonstrates that at least one of these factors is required for determining the myogenic lineage during embryonic development and activation of *myogenin* and *MRF4* are dependent upon the preceding expression of MyoD and/or Myf5 (60).

Gene targeting of the *myogenin* locus provided the first indication of the importance of the MRFs during development. In accordance with the appearance of myogenin at the onset of differentiation, lack of myogenin leads to perinatal death due to a severe deficiency of differentiated muscle fibers in newborn mice (61,62). Areas of presumptive muscle development have normal numbers of myonuclei and these cells are capable of differentiation when cultured *in vitro* (62). Interestingly, closer examination of *myogenin* null mice indicates that primary muscle fiber formation is unaffected whereas there are defects in secondary fiber myogenesis (63).

Three laboratories inactivated the *MRF4* gene yielding a range of defective rib cage phenotypes (64,65,66). The severity of the rib phenotype correlates with perturbations in *Myf5* expression, which lies approximately 6 kilobases away suggesting cis-regulatory elements (67,68). The most severe rib defects are observed in mice that do not activate *Myf5* (64). Moderate perturbation of *Myf5* expression leads to alterations in myotomal muscle development and rib abnormalities (65). Mice with normal *Myf5* expression are born healthy and fertile with minor rib abnormalities and show a four-fold increase in *myogenin* expression (66,69). This suggests increases in myogenin levels are able to compensate for the lack of MRF4.

Together, the gene targeting experiments suggest a model (Figure 2) in which MyoD and Myf5 act to determine the myoblast lineage whereas myogenin and MRF4 are important for differentiation and maintenance of the terminally differentiated state (70,71).

To further understand the functional relationships of the MRFs, mice lacking multiple MRFs or, mice in which the coding sequence of one MRF has been knocked-in to the locus of another, have been examined. Mice lacking functional copies of both *myogenin* and *MyoD*, *myogenin* and *Myf5*, *myogenin* and *MRF4* or lack all but *Myf5* are phenotypically identical to *myogenin* null mice indicating that myogenin is genetically downstream of both MyoD and Myf5 (69,72,73). Surprisingly, mice lacking *MRF4* and *MyoD* yield a phenotype similar to that of myogenin null mice (72). This indicates that myogenin can only compensate for the lack of MRF4 in the presence of MyoD expression lending support to the hypothesis that that different lineages are defined by MyoD and Myf5 expression. Furthermore, the data suggests that MyoD and myogenin cooperate to define one lineage whereas Myf5 and MRF4 define a distinct lineage.

Substitution of the coding region of myogenin into the *Myf5* locus ($Myf5^{myg\text{-}ki}$) rescues the rib defect in a *Myf5* null background (74). However, mice homozygous for $Myf5^{myg\text{-}ki}$ in a *MyoD* null background die perinatally due to reduced muscle formation. Furthermore, $Myf5^{myg\text{-}ki}$ in a *myogenin* null background are born with a *myogenin* null phenotype showing that the early expression of myogenin is unable to compensate at later time points of differentiation (75). It has been suggested that myogenic deficiencies observed in some multiple knock-out animals demonstrates that a critical threshold level of MRF expression is required to initiate terminal differentiation.

To obtain a greater understanding of how MyoD and Myf5 serve to determine lineages within the developing myotome, our laboratory examined the expression patterns of two transgenes that drive the expression of the bacterial beta-galactosidase (*lacZ*) gene under control of *MyoD* promoter elements. The upstream MD6.0-*lacZ* (6.0 kilobases of upstream *MyoD* promoter sequence) is detected in differentiated myocytes (76), whereas the 258/-2.5*lacZ* transgene (which has the 258 base pair –20 kilobase core enhancer fused to 2.5 kilobases of the *MyoD* promoter) is detected in determined myoblasts (77).

Mice lacking *Myf5* demonstrate a 2.5 day delay in development of paraspinal, intercostal and limb muscles (78,79), confirming previous reports that delayed expression of *MyoD* in a *Myf5* null background marks the onset of muscle differentiation (80). By contrast, mice lacking *MyoD* demonstrate normal epaxial muscle formation while hypaxial muscle development is delayed approximately 2 days (78,79). These results provide strong evidence that epaxial musculature is dependent upon Myf5 expression whereas MyoD is required for appropriate hypaxial muscle formation (81).

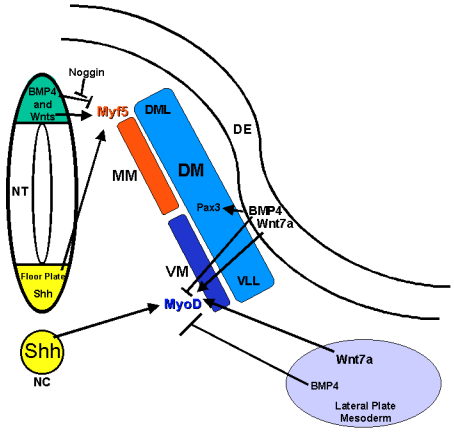

Figure 3: Extracellular growth factors which are important for myotomal development. Sonic hedgehog is secreted by both notochord and floor plate which serves to induce *Myf5* expression. Wnts, in particular Wnt1, secreted from the dorsal neural tube similarly induce *Myf5* expression in the epaxial myotome. By contrast, Wnt7a secreted from the dorsal ectoderm induces *MyoD* expression in the ventral myotome. BMP4 secreted from the dorsal ectoderm and lateral plate mesoderm is important for repressing MRF activation and maintaining *Pax3* expression in cells of the dermomyotome and the migrating precursor population in the VLL. Both dorsal neural tube and the DML secrete noggin, inhibiting the repressive effects of BMP4 on myogenesis. DML=dorsomedial lip; VLL=ventrolateral lip; DE=dorsal ectoderm; NT=neural tube; NC=notocord; MM=medial myotome; VM=ventral myotome.

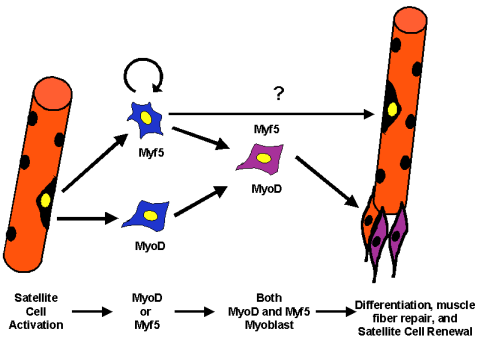

Figure 4: Activation and self-renewal of satellite cells. Satellite cells are activated by trauma or weight bearing. Initially, cells express *MyoD* or *Myf5* and proliferate. Expression of both *MyoD* and *Myf5* is found in cells prior to differentiation and fusion onto the existing damaged fiber. Studies examining the role of MyoD during muscle regeneration suggest that Myf5 expression may play a role in the self-renewal capacity of the satellite cell compartment since cells lacking MyoD do not exit the proliferative phase of activation and demonstrate a diminished capacity for differentiation. See text for details.

To examine whether the migratory hypaxial population of cells are affected in the absence of MRF expression, mice lacking both *MyoD* and *Myf5* were examined using the 258/-2.5*lacZ* transgene (82). Expression of *lacZ* is detected in both newly formed somites and limb buds. This pattern of staining demonstrates that in the absence of MRF expression activation of the –20 kilobase enhancer of MyoD occurs and cell migration to the limbs is unaffected (82). Interestingly, many *lacZ* positive cells in both the somitic and limb bud regions adopt non-myogenic fates suggesting these cells are multipotential (82). This confirms reports demonstrating that in the absence of Myf5, cells migrate abnormally and have an increased propensity to terminally differentiate along non-myogenic cell fates (83). The importance of Myf5 for certain myogenic lineages is strengthened by the fact that smooth muscle cells of the esophagus are delayed in their transdifferentiation to skeletal muscle in the absence of Myf5 expression (84).

Taken together, the data obtained from transgenic mice clearly demonstrates that MyoD and Myf5 are responsible for the determination of two distinct populations of muscle cells in the myotome. However, the precise mechanisms involved with initiating the expression of *MyoD* versus *Myf5* remains unclear.

3.3. Regulation of myogenesis during development

3.3.1. Extracellular cues regulating myogenic determination

Several factors are expressed in axial and lateral regions of the developing embryo which are important for somite formation and the determination of cell lineages (Figure 3; for review see 85). Axial structures, such as the neural tube and notocord, provide signals necessary for epaxial myogenic determination (86-91). By contrast, the hypaxial myogenic lineage is dependent upon signals originating from the lateral plate mesoderm and dorsal ectoderm (92-97). Factors secreted from these structures include sonic hedgehog (Shh), Wnts, transforming growth factor–beta (TGF-beta)-like molecules, fibroblast growth factors (FGFs) and the bone morphogenic proteins (BMPs). All of these factors regulate myogenic determination and differentiation. However, there are differential effects observed between epaxial and hypaxial musculature.

Sonic hedgehog (Shh) is expressed in the notochord and neural floor plate and has been shown to positively regulate the formation and survival of the dorsal myotome (98-101). Mice lacking *Shh* show reduced *Myf5* expression in the expaxial myotome (102,103), however, formation of the hypaxial myotome and *MyoD* expression is unaffected (103). In association with Shh, several Wnts have been shown to induce myogenesis and are thought to synergistically act with Shh (99,104,105). Mice lacking both *Wnt-1* and *Wnt-3a* are unable to from the medial dermomyotome but show normal development of the lateral myotome (106). Interestingly, Wnt-1 induces Myf5 expression whereas Wnt-7a, which is expressed in the lateral plate mesoderm, induces *MyoD* expression (107). These results confirm previous studies demonstrating that the neural tube induces *Myf5* expression while the dorsal

ectoderm preferentially activates *MyoD* expression (94). Taken together, this data indicates the importance of Shh and Wnt signaling during development. Moreover, it confirms the hypothesis that epaxial and hypaxial musculature represent distinct lineages dependent on Myf5 and MyoD expression, respectively.

The BMPs belong to the TGF-beta family of secreted factors and information obtained thus far shows these factors negatively regulate myogenesis. In particular, BMP4 has been of some interest due to its high level of expression in the lateral plate mesoderm (93). Recent experiments looking at the effects of BMPs on cells strongly suggest BMP concentration gradients are vital for cells to respond appropriately (108). Low BMP levels in the limb bud maintain migrating, *Pax3* expressing myogenic precursor cells in a proliferative state and repress myogenesis. By contrast, high BMP concentrations induce cell death (108). Important aspects of BMP signaling are the patterns of expression of BMPs and their inhibitors follistatin, noggin and chordin. Expression of the BMP antagonist, noggin, in the DML and lateral plate regulates the development of both medial and lateral myogenic lineages (105,109,110). Indeed, ectopic expression of noggin in the lateral regions of the embryo represses *Pax3* expression, expands the *MyoD* expression domain, and induces myogenesis (110).

Several FGF and TGF-beta family members have been identified. Treatment of cultured myoblasts with these factors suggests they act to stimulate proliferation and repress terminal differentiation. However, *in vivo* these molecules are important for the formation and terminal differentiation of the dorsal myotome (111). Neutralizing antibodies to TGF-beta or basic-FGF (bFGF) inhibit myotomal induction by axial structures. Exposure of segmental plate explants to a combination of TGF-beta and bFGF induces myotome formation. TGF-beta acts to specify the cells to the myogenic lineages whereas bFGF acts to promote proliferation and cell survival (111). Other TGF-beta and FGF molecules have been shown to play a role during regeneration and these will be discussed below (Section 3.5.1).

Cell-cell contact during development represents an important mechanism that contributes to the formation of distinct cell types. The transmembrane proteins of the *Notch-Delta/Jagged* signaling pathway are involved with cell contact signaling (112). Upon interaction of a Notch expressing cell with a Delta/Jagged expressing cell, the intracellular portion of Notch is cleaved, translocates to the nucleus and suppresses differentiation. Overexpression of the cytoplasmic portion of Notch represses myogenesis (113). During development, *Notch2* is expressed in cells of the DML, which lie juxtaposed to *Delta* expressing cells in the developing somite (114,115). This suggests that Notch2 suppresses myogenic commitment prior to cells extending beneath the dermomyotome.

3.3.2. Genes important for myoblast migration during development

Migration of cells from the VLL to the developing limb buds is required for the formation of limb hypaxial musculature. The naturally occurring *splotch* mutant mouse does not develop limb musculature (116). This is due to a loss-of-function mutation in the *Pax3* gene which is required for cells of the VLL to migrate (117-119). It should be noted that although migration of muscle precursor cells is impaired, transplantation of these cells from the VLL to the limb bud shows they are capable of terminal differentiation (120). Overexpression of Pax3 in cells represses myogenesis suggesting that it is involved with maintaining migrating myoblasts in an undifferentiated state (108). Indeed, upregulation of *Pax3* occurs in cells exposed to BMP signals from the dorsal ectoderm and limb buds, thus permitting muscle precursor cells to migrate and proliferate prior to differentiation (93,108,121).

Although cells that do not migrate in *splotch* mice are specified to the myogenic lineage there is evidence that Pax3 is involved with determination of the myogenic lineage. Generation of mice lacking *Myf5* in a *splotch* background demonstrates a surprising genetic relationship between *Pax3*, *MyoD* and *Myf5* (122,123). *Splotch* mice demonstrate normal myotomal development and activation of *MyoD*. However, *splotch* mice lacking *Myf5* do not form any musculature due to a lack of *MyoD* expression in the developing somite (122,123). Moreover, exposure of paraxial mesoderm explants to Pax3 can induce myogenic differentiation, supporting a role for Pax3 in activating MyoD in a Myf5 independent pathway during somitogenesis (122).

The c-Met receptor tyrosine kinase and its cognate ligand hepatocyte growth factor/scatter factor (HGF/SF) are important for the migration of myogenic cells. Targeted disruption of the *c-Met* or *HGF/SF* genes leads to a similar phenotype as that observed in *splotch* mice (124,125). Similar to *splotch* mice, there are not any defects in myotomal development. Moreover, although migratory cells of the VLL do not delaminate and migrate to the limbs, they are specified to the myogenic lineage, as observed in *splotch* mice (124,126).

Lbx1 is a homeobox protein expressed in the VLL and in *Pax3* positive migrating cells. Targeted inactivation of *Lbx1* leads to a disruption of only a subset of forelimb muscles and complete ablation of hindlimb musculature (127-129). Specifically, forelimb extensor muscles are absent, implicating Lbx1 in the dorsoventral migration pattern of myogenic precursor cells during development (127-129). Interestingly, *Lbx1* expression is not detected in the trunk-level dermomyotomes of *splotch* mice suggesting that in certain regions of the developing embryo, Pax3 is involved with activation of Lbx1 expression (130).

Msx1 is a homeodomain protein that demonstrates overlapping expression with *Pax3* and represses myogenesis *in vitro* (131). Interestingly, Msx1 has recently been shown to be antagonistic to both *Pax3* and MRF expression. This regulation is mediated by direct interaction between Msx1 and Pax3, blocking Pax3 DNA binding, and is important for controlling the timing of myogenesis in the limb (132,133).

The molecules responsible for activating MRF expression during development are currently unknown. However, recent studies examining the expression and activity of Dach2, Eya, Six1 and Pax3 proteins have suggested one mechanism by which the MRFs may be activated in the myotome (134,135). These four molecules are expressed in the dermomyotome, myotome and the migratory population of cells in the VLL (135). Dach2 and Pax3 positively autoregulate the expression of each other and myogenesis is induced within the somite by expression of Dach2/Eya2 or Six1/Eya2 complexes (134). Although it is not known whether these transcription factor complexes activate the promoters of *MyoD* or *Myf-5* directly, it is clear that these proteins are likely responsible for the ability of ectopic Pax3 expression to induce myogenesis in non-muscle tissue (122).

3.4. Regulation of terminal differentiation

3.4.1. Cell cycle and myogenesis

Decreases in growth factor concentration represents a cue for myoblasts to exit the cell cycle and undergo terminal differentiation. As myoblasts exit the cell cycle, expression of cyclin/cdk (cyclin-dependent kinase) inhibitors and retinoblastoma protein (pRb) are upregulated (135-138). The importance of cyclin/cdk inhibitors and pRb has been demonstrated by the fact that overexpression of E1A, which renders pRb inactive, inhibits myogenesis and can permit terminally differentiated myotube nuclei to reenter S-phase (139,140). Similarly, myoblasts that lack a functional copy of *Rb* re-initiate DNA synthesis upon growth factor stimulation. However, unlike E1A mediated inactivation of pRb, *Rb* null cells are capable of differentiating in the absence of pRb suggesting that p130 and p107 can compensate during differentiation but, are unable to maintain the differentiated phenotype (141,142). Moreover, both MRF and MEF2 proteins are dependent on pRb expression for full gene activation (143,144).

Overexpression of cyclin D1, which is important for the G1-S transition, and increases in cyclin/cdk kinase activity inhibit myogenesis, possibly due to phosphorylation and destabilization of MyoD (145-147). The putative phosphorylation residue is serine-200 which, when mutated to alanine, leads to an increase in MyoD stability and activity (148). Furthermore, MyoD and Myf5 protein level oscillations during the cell cycle correlate with changes in cyclin expression and cyclin/cdk activity (149). Physiologically, cyclin D1 levels increase upon stimulation of myoblasts with tumor necrosis factor alpha (TNF-alpha), leading to inhibition of terminal differentiation (150).

During terminal differentiation, upregulation of cdk inhibitors is important for cell cycle withdrawal (151), resistance to apoptosis (152), MyoD stability (153) and for the induction of myogenin, which is necessary for the differentiation program to proceed (154). Recent data demonstrates a direct link between MyoD and cell cycle regulation (155,156). In proliferating myoblasts nuclear cdk4 binds MyoD and inhibits MyoD-mediated gene expression (155). Conversely, a short carboxyl-terminal sequence of MyoD can inhibit cyclin/cdk4-dependent phosphorylation of pRb, promoting terminal differentiation (156). Furthermore, upregulation of the cyclin/cdk inhibitor $p57^{KIP2}$ stabilizes MyoD by blocking cyclinE-cdk2 activity (153) and by direct interaction with MyoD (157). It is clear that a fine balance exists between cell cycle regulation and terminal differentiation.

3.3.2. The Mef2 family of transcription factors

Along with the MRFs, it has been suggested that the myocyte enhancer factor 2 (MEF2) family of transcription factors play a role in myogenesis (for review see 158,159). MEF2 proteins are members of the MADS (MCM1, agamous, deficiens, serum response factor) box-containing family of transcription factors. The MEF2 family consists of four members, MEF2A-D, and they demonstrate a widely distributed pattern of expression. Although much of the information regarding these factors demonstrates their importance in cardiac muscle, they have been shown to form autoregulatory loops with the MRFs and are important for the expression of many muscle-specific genes. Structurally, MEF2 proteins are composed of amino terminal MEF and MADS domains which are responsible for dimerization and DNA binding. The carboxyl terminal domains thought to be important for gene activation and kinase responsiveness (Black and Olson, 1998). Homo- and heterodimers bind an A/T rich DNA sequence element ($C/TTA(A/T)_4TAG/A$) which is found in the promoters of many muscle-specific genes (158).

Several lines of evidence suggest that MEF2 and MRFs synergistically activate gene expression. It is important to note that MEF2 expression is initiated after the onset of differentiation suggesting these factors are involved during later stages of terminal differentiation (159). At the level of gene expression, full activation of both *MRF4* and *myogenin* promoters require both MRF and MEF2 proteins (160,161). *In vitro*, MRF and MEF2 proteins are capable of interacting to activate gene expression by both indirect and direct mechanisms (162,163). In flies, ablation of the single *MEF2* gene results in an inability of muscle cells to differentiate (164). By contrast, targeted inactivation of the *MEF2C* gene in mice is embryonic lethal due to severe defects in cardiac morphogenesis (165). However, no defects in skeletal muscle were noted, possibly due to functional redundancy of the factors. Transgenic mice carrying a *lacZ* reporter gene regulated by MEF2 factors show that MEF2 activity is high during embryonic development but is not detected after birth (166). Downregulation of MEF2 activity suggests that MEF factors are regulated at a posttranslational level that is currently unknown (166).

3.4.2. Growth factors and signal transduction

The determination, maintenance and activation of the myogenic program during development is regulated by factors such as Shh, BMPs, FGFs and Wnts. To gain an understanding of how extracellular signals regulate myogenesis, several studies have been carried out using myoblast cell lines *in vitro*. Treatment of cells with growth factors and cytokines leads to the activation of several intracellular kinase pathways which ultimately lead to changes in gene expression, cell survival and cellular morphology (for review see 167). Many distinct

mechanisms have been elucidated to explain how growth factors are able to repress or stimulate the myogenic program.

Protein kinase C (PKC) activity is increased in response to mitogenic stimulation. Overexpression of activated PKC represses MRF-mediated transcription of muscle-specific reporter vectors and terminal differentiation. Transcriptional activation and DNA-binding are regulated by the direct phosphorylation of a threonine residue in the basic domain of myogenin (168). Although this threonine residue is conserved in all four MRFs, PKC phosphorylation is specific for myogenin suggesting that PKC-mediated regulation of myogenesis involves other pathways (169).

Binding of ligands to cell-surface receptors initiates a cascade of events which leading to the activation of $p21^{ras}$. Overexpression activated $p21^{ras}$ in 10T1/2 mouse fibroblasts inhibits MRF-mediated differentiation without altering DNA-binding or the inherent transcriptional activation properties of the MRFs (170). Interestingly, inhibition of MEK and rac/rho kinase pathways, which are activated by ras, do not rescue myogenesis suggesting these pathways are not involved in regulating terminal differentiation (171). However, more recent studies demonstrate that inhibition of the MEK signaling pathway alleviates the repressive effects of FGF on myoblast differentiation (172). Furthermore, overexpression of the MAPK phosphatase, MKP-1, which is normally upregulated during differentiation, is important for inhibiting MAPK activity and permitting differentiation (173). It should be noted that later stages of differentiation require MKP-1 downregulation for myoblast fusion and myotube formation (173). Taken together, it is clear that increases in MAPK signaling are required for transmitting growth signals and decreases in MAPK activity is required for myogenesis to proceed.

Insulin-like growth factors (IGFs) are known to positively regulate myogenesis. IGF stimulation leads to an increase in phosphatidylinositol 3-kinase (PI3'K) activity. Dominant negative forms of PI3'K or, inhibition of PI3'K activity using synthetic inhibitors, are able to block IGF-mediated differentiation (174-176). When IGF signaling is blocked, cells maintain high levels of Id proteins and are unable to upregulate $p21^{Cip1}$ for cell cycle withdrawal (174). Conversely, expression of activated PI3'K is able to induce differentiation suggesting a direct role for PI3'K in myogenesis (176).

The lipid products resulting from stimulation of PI3'K activity serve to activate protein kinase B (PKB/Akt). During differentiation, PKB expression is upregulated and its activity is important for myocyte survival (177). Expression of a dominant negative form of PKB inhibits PI3'K and IGF stimulation of myogenesis indicating PKB lies downstream of these signals (178). Surprisingly, activated PKB is able to phosphorylate Raf, rendering the Raf/MEK/MAPK pathway inactive (179). Although this inhibition is important during differentiation, overexpression of activated PKB is unable to force differentiation under growth conditions suggesting the involvement of mediators that are specifically expressed at the onset of myogenic differentiation (180).

In many cell lines, the absence of extracellular growth factor stimulation leads to apoptosis indicating that pathways exist that are essential for cell survival. Although platelet-derived growth factor (PDGF) and IGF elicit opposite responses in myoblast cell lines, either factor on its own is sufficient to prevent apoptosis (181). Two distinct pathways are utilized indicating that cell survival can be mediated by separate mechanisms (181). What is surprising is that myoblasts stimulated with PDGF, which is mitogenic, produce a transient PKB activation and prolonged ERK activation. By contrast, IGF leads to transient ERK activation and prolonged PKB activity suggesting that the decision to proliferate is dependent upon the length of time that the MAPK pathway is active (181). This mechanism has been proposed for regulating proliferation versus differentiation decisions in the pheochromocytoma cell line, PC12, although prolonged ERK activity leads to differentiation (182). It remains to be seen what molecular events occur in myoblasts to elicit these distinct responses to extracellular cues.

MEF2 proteins are positively regulated by both p38 stress-activated and MKK5/BMK1 kinase pathways (183-186). The finding that MEF2 factors represent downstream targets of these pathways suggests that activation of MEF2 transcriptional activity is an important step during myogenesis. Indeed, overexpression of p38 isoforms or, upstream activators, stimulates myogenesis (187,188). It is interesting to note that the gamma isoform of p38 (SAPK3-beta/ERK6) is highly expressed in skeletal muscle. Although expression of this kinase is upregulated upon differentiation, it does not appear to phosphorylate MEF2 proteins and therefore, its function remains unclear (187,189).

Slow and fast muscle fibers differ in their metabolic properties and the panel of contractile proteins that they express. Since intracellular levels of calcium are regulated by contraction speeds, it has been hypothesized that calcium activated signal transduction pathways are important for fiber-type specification (159). Calcineurin, which is a calcium-activated protein phosphatase, activates the NFAT (nuclear factor of activated T-cells) transcription factors by dephosphorylation. This permits nuclear translocation of NFATs where they interact with other transcription factors and activate gene expression (190). Interestingly, treatment of animals with cyclosporin A, an inhibitor of calcineurin, or overexpression of calcineurin in muscle causes a shift from fast to slow fibers (191-193). One potential mechanism by which NFATs are thought to alter fiber-type specific gene expression is by interaction with MEF2. Response of T-cells to changes in intracellular calcium levels is mediated by MEF2 proteins (194,195,196) and many fiber-type specific gene promoters contain both MEF2 and NFAT binding sites (159).

3.4.4. Functional protein-protein interactions

Growth factor stimulation increases AP-1 (fos/jun)-dependent gene expression. Expression of the *c-fos* gene is mediated by binding of the serum-response-

factor (SRF) to a serum-response-element (SRE) in the c-fos promoter. During differentiation, *c-fos* gene expression is downregulated leading to decreases in AP-1-meditated gene activation. MRF-mediated repression of *c-fos* expression requires an E-box element that overlaps the SRE in the *c-fos* promoter (197). It is unclear whether MRF-mediated repression represents a competition for binding sites or, if direct interaction between MRFs and SRF is required (198). Moreover, the finding that jun can interact directly with MyoD and inhibit MRF-mediated gene expression suggests that AP-1 and MRFs form an autoregulatory loop to control myogenesis (199,200).

MRFs require dimerization with E-proteins in order to bind DNA and activate gene expression. One potential level of regulation involves the Id factors. Id molecules contain a helix-loop-helix motif but lack a basic DNA-binding domain (201 and references therein). Id levels increase upon stimulation of cells with growth factors and dimerization of Id proteins with MRFs or E-proteins prevents DNA binding and MRF-mediated gene expression. Expression of a MyoD-E47 fusion protein is resistant to Id regulation demonstrating the functional significance of Id proteins in regulating MRF-mediated gene expression and terminal differentiation (202). Alterations in E-protein availability has also been shown to occur by the MyoR bHLH factor (203). MyoR is expressed specifically in skeletal muscle and its expression is downregulated upon differentiation. Unlike Id/E-protein dimers, MyoR/E-protein dimers bind DNA and serve to repress gene expression (203).

Although the Mos protooncogene is generally regarded as an upstream activator of the MAPK signal transduction pathway, activation of the Mos protooncogene in muscle cells stimulates myogenesis (204). Mos-mediated myogenic stimulation occurs at two levels. First, activated Mos stimulates dimerization of MyoD and E12 and second, MyoD directly interacts with Mos, inhibiting downstream Mos-mediated activation of the MEK/MAPK pathway (205,206). These results suggest that alterations in the dimerization status of the MRFs are important levels of myogenic regulation. Indeed, interaction of MRF/E-protein dimers with muscle LIM protein dramatically increases MRF/E-protein gene activation and stimulates myogenic differentiation (207).

Several molecules have been shown to interact with MyoD. Of particular interest is the regulation of MyoD activity by p300/CBP and PCAF. These molecules are vital for gene activation by altering the acetylation status of histone cores in DNA (208). The transactivation domain of MyoD and the MADS domain of MEF2 proteins interact with p300, which initiates cell cycle arrest and differentiation (209,210). Interestingly, the histone acetyltransferase (HAT) activity of p300 is dispensable for MRF-mediated gene expression and only serves to attract PCAF to the promoters of muscle-specific genes (211). Significantly, MyoD transcriptional activation requires the acetylation of several lysine residues located just amino terminal of the basic DNA-binding domain by PCAF (212). In light of the fact that under growth conditions MyoD interacts with N-CoR (213), this suggests a molecular switch during activation of the myogenic program. Under proliferating conditions, MyoD association with N-CoR serves to attract histone deacetylases (213). As differentiation proceeds, N-CoR levels decrease and p300/PCAF complexes initiate MyoD-meditated gene expression. Indeed, the fact that MyoD has two domains necessary for chromatin remodeling lends support to this type of regulation (47).

3.5. Regeneration of adult skeletal muscle

3.5.1. Satellite cells

In adult muscle, approximately 5% of the myonuclei present in muscle fibers represent satellite cells (214). Normally, these cells are mitotically quiescent but can be induced to proliferate due to stresses, such as physical trauma or weight-bearing (for review see 215,216). Activated satellite cells undergo multiple rounds of cell division, exit the cell cycle and fuse onto the existing damaged fibers (215). Several potential factors exist within the area of damage that may serve to activate satellite cells (216). Single-cell RT-PCR (reverse-transcription polymerase chain reaction) experiments show that quiescent satellite cells do not express detectable levels of MRFs, but do express the *Met* receptor and the muscle cell adhesion molecule *M-cadherin* (217). Upon activation, cells express either *MyoD* or *Myf5*, but eventually express both prior to progression through the differentiation program (217). What is unclear is how the satellite cell compartment is renewed.

Insight into satellite cell renewal has come from experiments examining the role of the MRFs during regeneration. Although *MyoD* null mice are born without apparent defects in skeletal muscle, when these mice are interbred with the *mdx* mouse or, adult muscle is subjected to damage, muscle regeneration is severely impaired even though several myogenic cells are detected in the damaged area (218). *In vitro* analysis of cells isolated from adult *MyoD* null mice demonstrate that these cells are unable to progress through the normal differentiation program and are mitotically active under conditions that initiate terminal differentiation in wild-type control cells (219,220). Although *MyoD-/-* cells express high levels of *Myf5*, their ability to terminally differentiate is impaired (219). Taken together, these results indicate that cells lacking MyoD may represent an intermediate phenotype between quiescent satellite cell and determined myogenic progenitor cell (mpc) (219,221). Moreover, the expression of Myf5 alone is insufficient for differentiation, suggesting that renewal of the satellite cell compartment may be a function of Myf5 expression (219,221).

More recent evidence suggests that the winged helix transcription factor MNF (myocyte nuclear factor) is essential for the maintenance of satellite cells. *MNF* expression is detected in quiescent satellite cells (222). Two alternatively spliced isoforms can be detected with the beta isoform expressed in quiescent cells and the alpha isoform in activated mpcs (223,224). Interestingly, mice lacking a functional copy of *MNF* show severe deficiencies in skeletal muscle regeneration and are unable to properly coordinate the

expression of cell cycle and myogenic determination genes (224). This suggests that MNF serves to properly activate genes responsible for determining mpcs and activating the myogenic program.

During regeneration, expansion of the mpc compartment is necessary for proper muscle repair to occur. FGF6 demonstrates a skeletal muscle-specific pattern of expression (225-227). Mice lacking *FGF6* are born healthy and fertile with no developmental abnormalities in skeletal muscle (228,229). However, these mice demonstrate a reduced capacity for regeneration after mechanical injury or when interbred with the *mdx* mouse (228). Although *FGF6* null mice have normal numbers of satellite cells, activation of the regeneration program yields fewer MyoD and myogenin positive cells and an increased deposition of collagen in sites of regeneration. This suggests that FGF6 normally represses differentiation and permits expansion of the satellite cell compartment during adult skeletal muscle regeneration (228).

By contrast, targeted inactivation of the TGF-beta family member, GDF8 or myostatin, results in mice with substantial increases in muscle mass (230). Both hyperplasia and hypertrophy are responsible for this increase in muscle mass. Unlike FGF6, GDF8 is a negative regulator of myoblast proliferation and differentiation and is involved with hypertrophic effects which is mediated by satellite cells (230).

Information from previous sections has demonstrated that members of the TGF-beta family are responsible for repressing the determination of myogenic cells whereas FGF family members stimulate proliferation of myogenic cells. Of interest are recent studies showing that both ski and sno oncoproteins are antagonistic to TGF-beta signaling (231,232). Overexpression of ski/sno proteins induces myogenesis and can mediate skeletal muscle fiber hypertrophy (233,234). This indicates that expression of ski and sno proteins may antagonize the negative effects of TGF-beta signaling on myogenic determination and the regeneration program.

3.5.2. Satellite cell origin

Early experiments using quail-chick chimeras suggested that satellite cells were somitically derived (25). These cells enter the limbs of mouse embryos at about day 17.5 p.c. (30,235,236). More recent analyses examining satellite cell origin suggest that this cell population may actually arise in the dorsal aorta of embryonic mice (236). Cultured cells isolated from the dorsal aorta coexpress skeletal muscle-specific and endothelial markers, similar to adult satellite cells. Moreover, these cells are able to contribute to regenerating muscle (236). Although *splotch* and *c-Met* null mice do not have cells migrate into the limb during development, cells isolated from the limbs of these embryos are myogenic, further supporting the notion that at least some satellite cells originate from the vascular system (236).

3.5.3. Stem-cells in skeletal muscle

To determine whether regenerating muscle recruits cells from non-satellite cell origin, genetically marked bone-marrow was transplanted into mice to assess the cellular contribution of bone-marrow to regenerating muscle (237,238). In both studies, it was shown that transplanted marrow cells are detected in regenerated fibers indicating that bone-marrow-derived-stem-cells contribute to skeletal muscle regeneration (237,238). Similar results are obtained when highly purified hematopoietic stem cells are injected intravenously into *mdx* mice (239). However, in all three cases, grafted cells do not contribute to the satellite cell compartment indicating that they are only capable of terminal differentiation (216).

To determine if muscle tissue contains stem cells similar to those found in bone marrow, cells isolated from adult muscle were subjected to a specialized FACS (fluorescence-activated cell sorting) technique (239). This method permits the isolation of stem cells on the basis of Hoescht 33342 dye exclusion (240,241). Analyses from adult skeletal muscle indicates that a population of stem-cells exists and these cells express muscle-specific markers *in vitro* (239). What is surprising is that intravenous injection of these cells indicates that not only are they capable of participating in muscle regeneration but they also contribute to the satellite cell compartment (239). Moreover, these cells also repopulate bone-marrow of lethally irradiated mice and give rise to the three major blood lineages (242). Similar studies have revealed that pluripotent stem-cells can be isolated from numerous different tissues, including neural tissue (243,244). The ability to isolate multipotential stem cells from numerous tissues may provide the raw material necessary for stem cell therapies for the treatment of several degenerative diseases including muscular dystrophies.

4. PERSPECTIVES

It is clear that a great deal of information has been obtained regarding many aspects of skeletal muscle development. The myogenic regulatory factors represent an ideal paradigm for the study of cell lineages and the molecular events required for the establishment of a terminally differentiated tissue. However, several questions remain concerning aspects of determination, proliferation and terminal differentiation. In particular, the molecules responsible for the *de novo* activation of *MyoD* and *Myf5* are unknown. Similarly, regulation of MRF activity during proliferation and terminal differentiation are poorly understood due to a lack of myoblast specific genes that have been identified to date. Although several signal transduction pathways and protein-protein interactions regulating MRF expression and activity have been described, how these processes are integrated represents a major challenge in muscle research. Moreover, coordination of cell cycle and terminal differentiation is complex and we are only now beginning to understand the **many** factors involved.

The recent investigations into satellite cell origin and the demonstration that multipotential stem-cells are readily isolated from adult tissue have raised a multitude of questions. The potential use of muscle-derived stem-cells for the treatment of many degenerative diseases is truly exciting. Research focussed on understanding the molecular nature of these cells and their potential uses will provide a great deal of insight into myogenesis.

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