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Antibody to MyoD Decreases Myogenin Gene Expression and Agrin-induced Acetylcholine Receptor Clustering

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Abstract

Objective: A family of myogenic regulatory factors, including MyoD and myogenin, guide myogenesis and neuromuscular synapse formation. Myogenin gene expression is activated by MyoD, and one of myogenin's functions is to activate gene expression of the acetylcholine receptor (AChR) at the neuromuscular synapse. Motor neurons release agrin as they near skeletal muscle fibers in development, which drives the clustering of existing AChRs to the site of neuromuscular synapse formation. We have previously demonstrated that continuous exposure to antibody to MyoD or myogenin decreases agrin-induced AChR clustering in C2C12 skeletal muscle cell culture. Our objective was to more specifically establish how MyoD and myogenin interact in development.

Methods: C2C12 cell cultures were exposed to experimental manipulations including antibodies to MyoD and myogenin, and myogenin morpholino. Endo-Porter was used to enhance cell uptake of experimental manipulations. AChR clustering assays were performed to assess the effect of antibody or morpholino on agrin-induced AChR clustering. Western blots were performed to assess myogenin gene expression after antibody or morpholino exposure.

Results: The results reported here demonstrate that exposure as short as eight hours for antibody to myogenin can decrease agrin-induced AChR clustering in myotubes. We have previously demonstrated that some experimental manipulations reduce myogenin gene expression concurrent with a decrease in agrin-induced AChR clustering. The current results establish more specifically how MyoD and myogenin interact in neuromuscular synapse formation by demonstrating that exposure to antibody to MyoD reduces myogenin gene expression concurrent with a decrease in agrin-induced AChR clustering.

Conclusion: These results suggest that MyoD is essential for agrin-induced AChR clustering through a mechanism that includes activation of myogenin gene expression, leading to activation of AChR gene expression, and ultimately production of an appropriate level of AChR for agrin-induced AChR clustering and neuromuscular synapse formation.

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Introduction

Myogenic regulatory factors MyoD (myf3), myogenin (myf4), myf5, and MRF4 (myf6) guide skeletal muscle development. They evolved from a single gene as a result of gene duplication events and subsequent mutations [1]. These basic helix-loop-helix transcription factors bind to the E-box found in the promoters or enhancers of many muscle-specific genes to activate or inhibit transcription [2-6]. Western blots using C2C12 cell culture verified *in vitro* the temporal expression pattern initially determined with experiments using knockout mice. MyoD was expressed in proliferating myoblasts and myotubes, while myogenin was primarily expressed in myotubes [7]. This expression pattern, where MyoD is expressed early in myogenesis and myogenin is expressed later in myogenesis, was also demonstrated in C2C12 cell culture using immunofluorescence [8]. Skeletal muscle cell cultures, such as the C2C12 cell line derived from mouse hindlimb, provide simplified systems for studying myogenesis and more specifically the development of the postsynaptic component of the neuromuscular synapse [9,10].

This postsynaptic component includes acetycholine receptors (AChRs), as well as other molecules that aggregate and co-localize with AChR, including a low-density lipoprotein receptor-related protein (Lrp4), a muscle-specific kinase (MuSK), and rapsyn. Activated motor neurons release acetylcholine, which binds AChRs as a first step in muscle fiber contraction. In early development AChRs cluster spontaneously but aggregation increases upon exposure to motor neuron derived agrin [11-13]. As motor neurons approach skeletal muscle fibers in development, agrin is released and binds to Lrp4, forming a complex of Lrp4 and MuSK which mediates MuSK activation by agrin [14-16]. Agrin binding stimulates tyrosine phosphorylation of MuSK [17] and Lrp4 [15], which drives a subsequent signaling pathway that includes tyrosine phosphorylation of AChRs leading to increased AChR clustering [18,19]. MuSK and rapsyn are essential for AChR clustering during neuromuscular synapse formation [17,20], with MuSK required for the signaling events that precede AChR clustering [17,21-23]. In addition, AChRs are required for the agrin-induced aggregation of MuSK at the neuromuscular synapse [24]. Suppression of Lrp4 gene expression decreases agrin binding activity, agrin-induced MuSK tyrosine phosphorylation, and agrin-induced AChR clustering [15].

In myogenesis, the expression of early genes is initiated by MyoD, while later expression of other genes is initiated by MyoD and myogenin. In the absence of MyoD, myogenin can inefficiently activate early genes [25]. In addition to increasing myogenin gene expression, MyoD also targets MuSK and rapsyn gene expression, while myogenin targets rapsyn but not MuSK gene expression [7]. Moreover, myogenin activates genes for AChR subunits [26,27]. Overexpression of myogenin in transgenic mice elevates mRNA and protein levels of all five AChR subunits, while reducing MyoD protein levels [28]. This suggests that myogenic regulatory factors

like MyoD and myogenin are intricately linked to the development of the postsynaptic component of the neuromuscular synapse. More specifically, MyoD has a role earlier in differentiation involving MuSK and rapsyn, while myogenin has a role later in differentiation involving rapsyn and AChR but not MuSK.

The C2C12 cell culture model has proven useful for asking fundamental questions concerned with muscle development and neuromuscular synapse formation, and is ideal for examining more precisely the roles of MyoD and myogenin in neuromuscular synapse formation. Using an immortalized rat muscle cell line, RNA interference experiments revealed that myogenin expression was necessary for robust spontaneous AChR clustering [29]. In C2C12 cell culture, antibody to either MyoD or myogenin decreased the frequency of agrin-induced AChR clustering without affecting myotube formation, demonstrating that MyoD and myogenin are necessary for agrin-induced AChR clustering [8]. The results in the present study establish more specifically how MyoD and myogenin interact in neuromuscular synapse formation by demonstrating that exposure to antibody to MyoD reduces myogenin gene expression concurrent with a decrease in agrin-induced AChR clustering. This suggests that MyoD is essential for agrin-induced AChR clustering through a mechanism that includes activation of myogenin gene expression, leading to activation of AChR gene expression, and ultimately production of an appropriate level of AChR for agrin-induced AChR clustering and neuromuscular synapse formation.

Methods

Cell culture maintenance

C2C12 myoblasts were derived from mouse hind limb (gift from H. Gordon, University of Arizona; [9,10], and are commonly used for skeletal muscle cell culture experiments. They are ideal for studying myoblast fusion to form myotubes, and acetylcholine receptor (AChR) clustering. For normal maintenance of C2C12 cell culture, myoblasts were first plated in growth medium (GM) on 10 cm plates at approximately 20% confluence. GM consists of Dulbecco's modified Eagle's medium (DMEM) plus 20% fetal bovine serum, 0.5% chick embryo extract and 100 U/ml streptomycin/penicillin. GM was replaced daily, and myoblast cultures were split at approximately 60% confluence into new plates. For formation of myotubes, myoblasts were plated in GM on 22x22 mm cover slips that had been flamed in 200-proof ethanol and placed in 6-well plates. GM was replaced daily. After 48 hours in GM, myoblast cultures typically reached 80% confluence, and cultures were then switched to differentiation medium (DM). DM consists of DMEM plus 2% horse serum and 100 U/ml streptomycin/penicillin. DM was replaced daily as myoblasts fused into myotubes, and cultures were maintained for 72 hours in DM. All cultures were exposed to 10 ng/ml agrin (R&D Systems) for the last 16 hours of 72 hours in DM to induce AChR clustering. The incubator was maintained at 37°C under 8% carbon dioxide and 100% humidity.

Experimental manipulations

C2C12 cell cultures were maintained as untreated controls or exposed to antibody to MyoD (Santa Cruz Biotechnology sc-71629) or antibody to myogenin (Santa Cruz Biotechnology sc-12732), or exposed to myogenin morpholino (Gene Tools), for various periods of time during the 72 hours in DM. To optimize antibody or mopholino delivery into cells, media with 6 μM Endo-Porter (Gene Tools) was added 24 hours prior to experimental treatments. Endo-Porter enhances an endocytosis-mediated process. Previously Endo-Porter was shown to specifically increase cellular uptake of antibody to MyoD or myogenin [8]. Antibodies were added at concentrations ranging from 1-5 μg/ml, and for periods of time ranging from 1 hour to 24 hours. Morpholinos were added at 1 μM for 24 hours. In all cases treatments occurred while cell cultures were in DM, and all cell cultures were fixed at 72 hours in DM. Previously concentrations of antibody to MyoD or myogenin as low as 1 μg/ml, added daily beginning when cell cultures were plated on coverslips in GM, was sufficient to decrease agrin-induced AChR clustering relative to untreated cultures [8].

Acetylcholine receptor clustering assay

AChRs were labeled by the binding of α-bungarotoxin conjugated to tetramethyl rhodamine (Molecular Probes; [30]). Cultures were incubated in the toxin-containing medium for 30 minutes at 37°C to label AChRs after 72 hours in DM. Cover slips were rinsed three times with room temperature phosphate buffered saline (PBS), fixed for 10 minutes with 2% paraformaldehyde in PBS, rinsed three times with PBS, dehydrated in cold methanol for 5 minutes at -20°C, and mounted on microscope slides in Vectashield Mounting Medium for Fluorescence (Vector Laboratories). AChR clusters were visualized with an IX70 Olympus inverted microscope under the 20X objective (yielding a total magnification of 200X), and fluorescent images were captured as high-resolution JPG files with an Olympus camera with Magnafire digital imaging software. Bright clusters of AChRs were observed on all aspects of myotubes in fluorescent images. AChR clustering was assayed in two ways. First, AChR clusters per image were determined by counting AChR clusters from randomly captured JPG files, with 25 JPG files captured from each cover slip, and 1-4 cover slips utilized for each data point (n=25-100). Files were coded and then AChR clusters were counted by an individual blind to treatment group. Second, total AChR clustered area was determined using an algorithm developed for Cell Profiler [31]. These data were utilized to assay agrin-induced AChR clustering with or without exposure to antibodies or morpholinos for MyoD or myogenin. Comparison of untreated cultures with exposed cultures were analyzed by Student's t-test to determine statistically different results at $p<0.05$, with the results presented as histograms. Representative images were assembled into figures.

Cell profiler algorithm

Each grayscale image was analyzed using Cell Profiler's object identification algorithm. The threshold used in the algorithm was defined as the minimum fluorescent intensity that a pixel must display to be counted as part of a cluster. Experimentation with the threshold revealed that a minimum brightness of 70% best defined a pixel with enough fluorescence to be counted as part of a cluster, and this threshold was used for all analyses. To ensure objectivity and consistent quantification, the threshold and all other settings were kept constant across all groups and images. The diameter range for identifying an AChR cluster was set at 4-150 pixels (2.93 μm–109.95 μm). Contiguous pixels meeting both the intensity and size requirements were counted as parts of AChR clusters. The total clustered pixels per image were displayed in histograms as total AChR clustered area (pixels), to quantify what number of pixels in an image was counted as containing clustered AChRs. Analysis of data trends via unpaired t-tests were completed using GraphPad Prism.

Western blots

To assay for protein levels of the myogenic regulatory factor myogenin, myotube cultures were divided into untreated cultures (controls), and those that had been exposed to 1 μg/mL or 5 μg/mL

antibody to MyoD for 24 hours beginning after 48 hours in DM. Myotube cultures were rinsed twice with calcium- and magnesium-free PBS (CMF-PBS), scraped off in RIPA complete lysis buffer (containing PMSF, sodium orthovanadate, and protease inhibitors), agitated for 30 minutes on ice, and then centrifuged at 13,000g for 2 minutes to create pellets containing insoluble materials such as organelles and extracellular matrix. The extracted supernatant was then frozen. At a later time samples were thawed, a BCA protein assay was performed to determine the concentration of protein in each sample, and samples were boiled for 5 minutes in sample buffer to reduce and denature proteins. Then samples were separated by electrophoresis on a 10% polyacrylamide gel (Bio-Rad) and transferred to a nitrocellulose membrane. The membranes were blocked with 5% milk in TBS-T, probed for 16 hours at 4°C with a mouse monoclonal antibody to myogenin (sc-12732; Santa Cruz Biotechnology) at 1:1000 in blocking solution, and then probed by a goat anti-mouse secondary antibody (926-32210; Li-Cor) at 1:10,000 in blocking solution for 30 minutes. The resultant western blot was then visualized with the Odyssey CLx, a near infrared imaging system. It uses solid state diode lasers to excite at 685 and 785 nm. For scanning, the western blot was placed on the glass surface of the odyssey and covered with a rubber mat. In the Li-Cor software a box was created to define the scanning area of the blot. The 700 and 800 channels were chosen and low quality image quality and 169 µm resolution was checked before start scan was pressed. As a loading control, all samples were also probed with a mouse monoclonal antibody to actin (sc-8432; Santa Cruz Biotechnology).

Results

Exposure to 5 µg/ml antibody to MyoD or myogenin for any 24 hour period in DM decreases AChR clustering

AChRs cluster spontaneously with a baseline frequency on C2C12 myotubes and this clustering is increased with agrin treatment [11-13]. C2C12 cell cultures were switched from growth medium (GM) to differentiation medium (DM) at 80% confluence, 10 ng/ml agrin was added for the last 16 hours in DM, and myotubes were examined for AChR clustering after 72 hours in DM. The results reported here demonstrate that when C2C12 skeletal muscle cell cultures were exposed to 5 µg/ml antibody to MyoD or myogenin, for any 24 hour period in DM, agrin-induced AChR clustering was decreased with statistical significance at $p<0.05$ using Student's t-test (Figures 1 and 2). Previously it was demonstrated that continuous exposure in GM and then for 72 hours in DM to 1 µg/ml antibody to MyoD or myogenin also decreased agrin-induced AChR clustering [8]. The current data show that a shorter exposure time can decrease agrin-induced AChR clustering if the antibody concentration is sufficient.

Exposure to 1 µg/ml antibody to myogenin, or 1 µM myogenin morpholino, for the first 24 hours in DM decreases AChR clustering

The results reported here demonstrate that when C2C12 skeletal muscle cell cultures were exposed to 1 µg/ml antibody to MyoD or myogenin, for any 24 hour period in DM, agrin-induced AChR clustering was only decreased with statistical significance at $p<0.05$ using Student's t-test when the exposure was to antibody to myogenin during the first or second 24 hours in DM (Figure 3). Exposure to 1 µg/ml antibody to myogenin during the final 24 hours in DM, or exposure to 1 µg/ml antibody to MyoD during any 24 hour period in DM, was insufficient to decrease agrin-induced AChR clustering. This contrasts with continuous exposure in GM and then for 72 hours in DM to 1 µg/ml antibody to MyoD or myogenin, which decreased agrin-induced AChR clustering [8]. These data show that the lower concentration of antibody to MyoD or myogenin, 1 µg/ml, was only sufficient to decrease agrin-induced AChR clustering if exposure was continuous, or if exposure to the antibody to myogenin occured during specific 24 hour periods. The C2C12 cell culture system appears to be more sensitive to antibody to myogenin than MyoD, and is especially sensitive when exposed to antibody to myogenin early in myotube formation.

The results reported here also demonstrate that exposure to 1 µM myogenin morpholino for the first 24 hours in DM, but not the last 24 hours in DM, decreased AChR clustering (Figures 4 and 5). This is

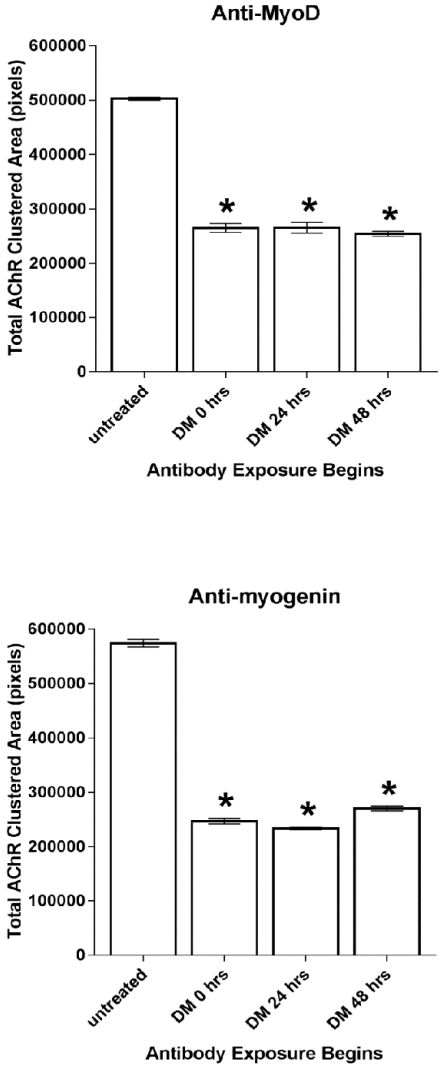

Figure 1: 5 µg/ml antibody to MyoD (A) or myogenin (B) for any 24 hour period in differentiation medium (DM) can decrease the frequency of agrin-induced AChR clusters. Clustering was quantified by analyzing fluorescent images from cultures that were untreated or exposed to 5 µg/ml antibody for a 24 hour period beginning with the introduction of DM, or after 24 hours in DM, or after 48 hours in DM. Cultures were fixed and images captured at 72 hours in DM. The histograms reveal that a 24 hour exposure to 5 µg/ml antibody to MyoD or myogenin at any time during differentiation was sufficient to decrease agrin-induced AChR clusters relative to untreated cultures, using Student's t-test at $p<0.05$. (*) statistically decreased from untreated cultures. Error bars indicate standard error of the mean (s.e.m.).

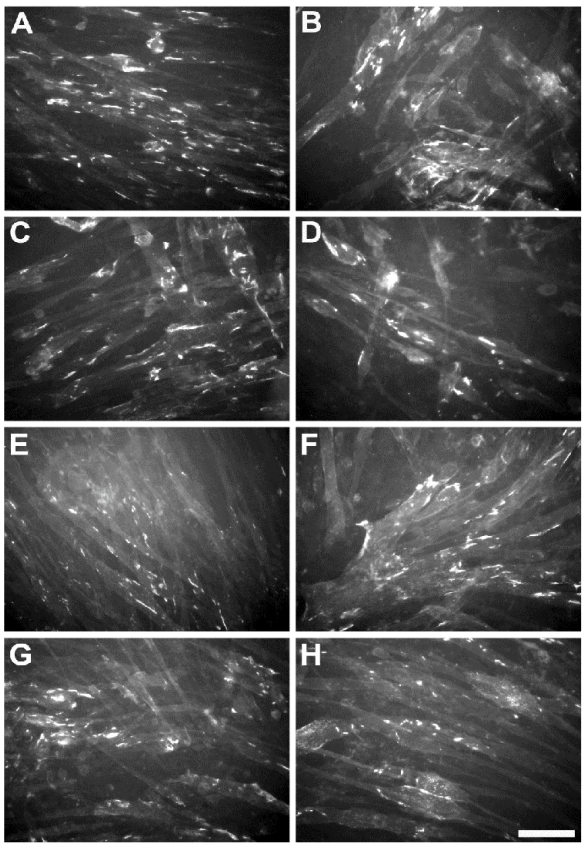

Figure 2: Example images of cell cultures exposed for 24 hours to 5 µg/ml antibody to MyoD or myogenin. Fluorescent images were captured from agrin-induced cultures that were untreated (A, B), or exposed to 5 µg/ml antibody to MyoD (C, E, G), or exposed to 5 µg/ml antibody to myogenin (D, F, H). The antibody exposure was for a 24 hour period beginning with the introduction of DM (C, D), or after 24 hours in DM (E, F), or after 48 hours in DM (G, H). Cultures were fixed and images captured at 72 hours in DM. Fluorescent areas are clusters of AChRs. Scale bar=100 µm.

a similar result to 24 hour exposure to antibody to 1 µg/ml myogenin, which decreased agrin-induced AChR clustering only when the exposure was in the first or second 24 hours in DM. The current data suggest that the C2C12 cell culture system is more sensitive to antibody or morpholino to myogenin when exposed early in myotube formation when myoblasts are first fusing into myotubes.

Exposure to 1 µg/ml antibody to myogenin for as brief as 8 hours can decrease AChR clustering

Exposure to 1 µg/ml antibody to myogenin during the first 24 hours in DM required a full 24 hours exposure to decrease agrin-induced AChR clustering. Exposure for only 8 hours or less was insufficient. If the exposure was during the second 24 hours in DM, an exposure of only 8 hours was sufficient to decrease agrin-induced AChR clustering, but exposure shorter than 8 hours was insufficient (Figure 6). The current data are consistent with other observations that the C2C12 cell culture system sensitivity to antibody to myogenin peaks early in myotube formation when myoblasts are first fusing into myotubes.

Exposure to antibody to MyoD decreases myogenin gene expression

Exposure to 5 µg/ml antibody to MyoD during the final 24 hours in DM decreased myogenin gene expression, but 1 µg/ml antibody to MyoD was insufficient (Figure 7). The current data confirm that MyoD is essential for normal myogenin gene expression, and suggests that decreases in agrin-induced AChR clustering are due to a decrease in myogenin, regardless of whether antibody to MyoD or myogenin is utilized. This is consistent with other observations and suggests

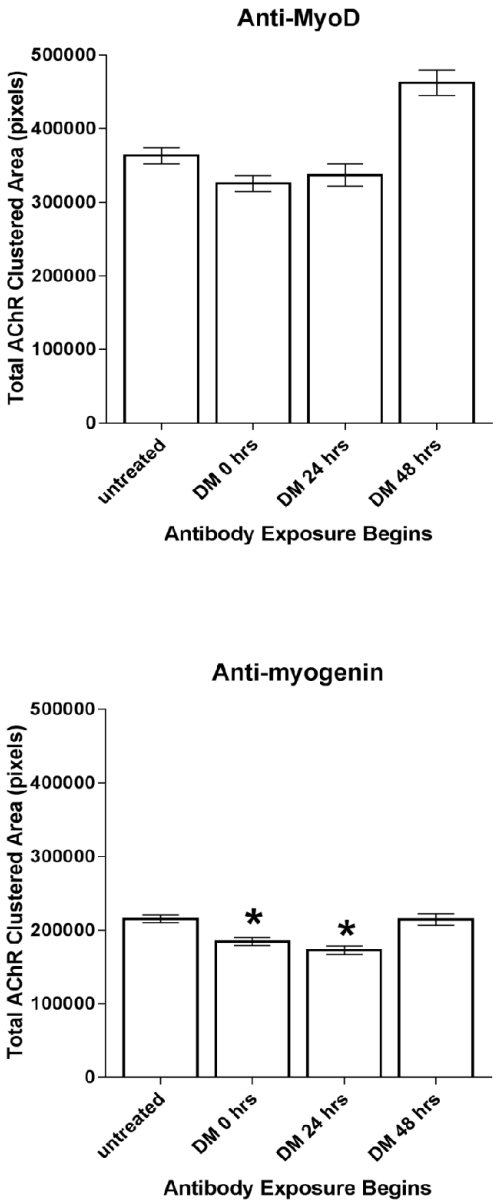

Figure 3: 1 µg/ml antibody to myogenin (B) but not MyoD (A) for some 24 hour periods in DM can decrease the frequency of agrin-induced AChR clusters. Clustering was quantified by analyzing fluorescent images from cultures that were untreated or exposed to 1 µg/ml antibody for a 24 hour period beginning with the introduction of DM, or after 24 hours in DM, or after 48 hours in DM. Cultures were fixed and images captured at 72 hours in DM. The histograms reveal that a 24 hour exposure to 1 µg/ml antibody to myogenin beginning with the introduction of DM, or after 24 hours in DM, was sufficient to decrease agrin-induced AChR clusters relative to untreated cultures, using Student's t-test at $p<0.05$. For other 24 hour time periods examined for myogenin, or for antibody to MyoD for any 24 hour time period, 1 µg/ml antibody was insufficient to have an effect. (*) statistically decreased from untreated cultures. Error bars indicate standard error of the mean (s.e.m.).

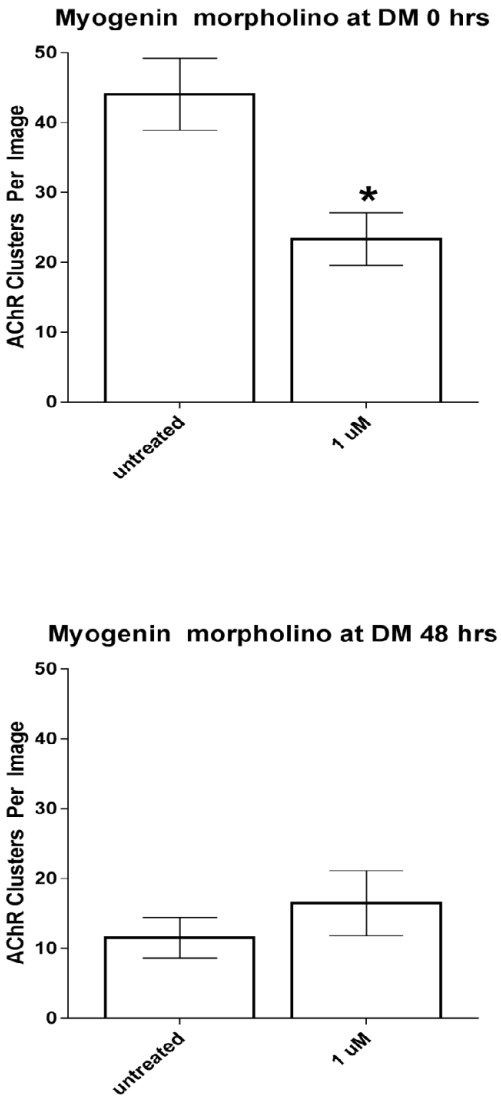

Figure 4: 1 μM myogenin morpholino for 24 hours can also decrease the frequency of agrin-induced AChR clusters. Clustering was quantified by analyzing fluorescent images from cultures that were untreated or exposed to 1 μM myogenin morpholino for a 24 hour period beginning with the introduction of DM, or after 48 hours in DM. Cultures were fixed and images captured at 72 hours in DM. The histograms reveal that a 24 hour exposure to 1 μM myogenin morpholino beginning with the introduction of DM (A) was sufficient to decrease agrin-induced AChR clusters relative to untreated cultures, using Student's t-test at $p<0.05$. Exposure to 1 μM myogenin morpholino for 24 hours beginning after 48 hours in DM (B) was insufficient to have an effect. (*) statistically decreased from untreated cultures. Error bar indicates standard error of the mean (s.e.m.).

that MyoD is essential for agrin-induced AChR clustering through a mechanism that includes activation of myogenin gene expression, leading to activation of AChR gene expression, and ultimately production of an appropriate level of AChR for agrin-induced AChR clustering and neuromuscular synapse formation.

Discussion

The temporal expression pattern of myogenic regulatory factors was initially determined with experiments using knockout mice. In the absence of myogenin early in somite formation, myotomes still form and myoblasts still appear [32]. Myoblast identity becomes established as long as MyoD and myf5 are present [33-35]. In contrast, myogenin is required for myoblast differentiation, as myoblasts proliferate and fuse together to form multinucleated myotubes [36,37]. Although MRF4 is highly expressed in adult skeletal muscle fibers it does not appear to be essential for myogenesis [38-42]. While single null mutations of MyoD, myf5, or MRF4 are not lethal [33,34,43], the null mutation for myogenin eventually results in severe muscle deficiency due to inadequate secondary muscle fiber development and subsequent neonatal death [32,36,37].

Myogenic regulatory factors are so specialized that expression of a single myogenic regulatory factor, MyoD, is sufficient to force nonmuscle cells to complete the myogenic program [44,45]. MyoD is considered the master myogenic transcription factor in triggering muscle terminal differentiation. Using tandem affinity purification coupled to mass spectrometry, the MyoD interactome was identified and defined [46]. MyoD interacts with core promoter factors in differentiation. Defective myogenic regulatory factors, or their core promoter factors, can lead to developmental muscle disorders such as congenital myasthenia and myotonic dystrophy.

Models of gene activation during myogenesis predict that genes are turned on sequentially beginning with the activation of myogenin by MyoD. An initial blueprint for myogenic differentiation combined genome-wide transcription factor binding and expression profiling to better elucidate the myogenic differentiation program in mammalian skeletal muscle cells. Myogenic regulatory factors direct myogenesis including the assembly of the neuromuscular synapse. Specifically, the initial blueprint demonstrated that in growing myoblasts MyoD targeted genes involved in synapse specification and neuromuscular function, whereas in myotubes MyoD and myogenin targeted genes involved in muscle development and contraction. Strikingly, transcription factors were the largest cluster of targets [7].

In the nucleus, MyoD and myogenin coordinate muscle-specific gene expression. In addition to activating myogenin, MyoD is involved in myoblast proliferation by establishing an open chromatin structure at muscle-specific genes. Myogenin drives high levels of

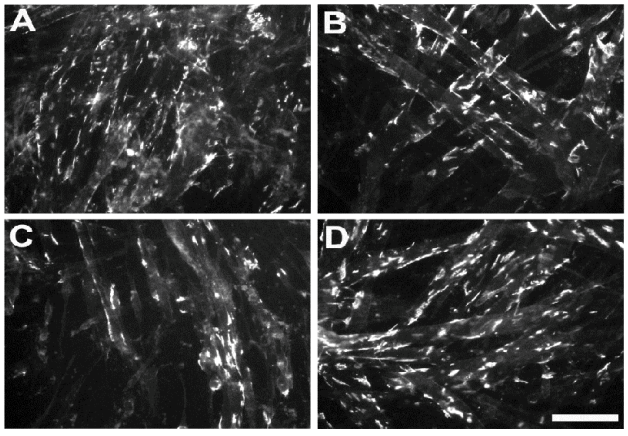

Figure 5: Example images of cell cultures exposed for 24 hours to 1 μM myogenin morpholino. Fluorescent images were captured from agrin-induced cultures that were untreated (A, B), or exposed to 1 μM myogenin morpholino for a 24 hour period beginning with the introduction of DM (C) or beginning after 48 hours in DM (D). Cultures were fixed and images captured at 72 hours in DM. Fluorescent areas are clusters of AChRs. Scale bar=100 μm.

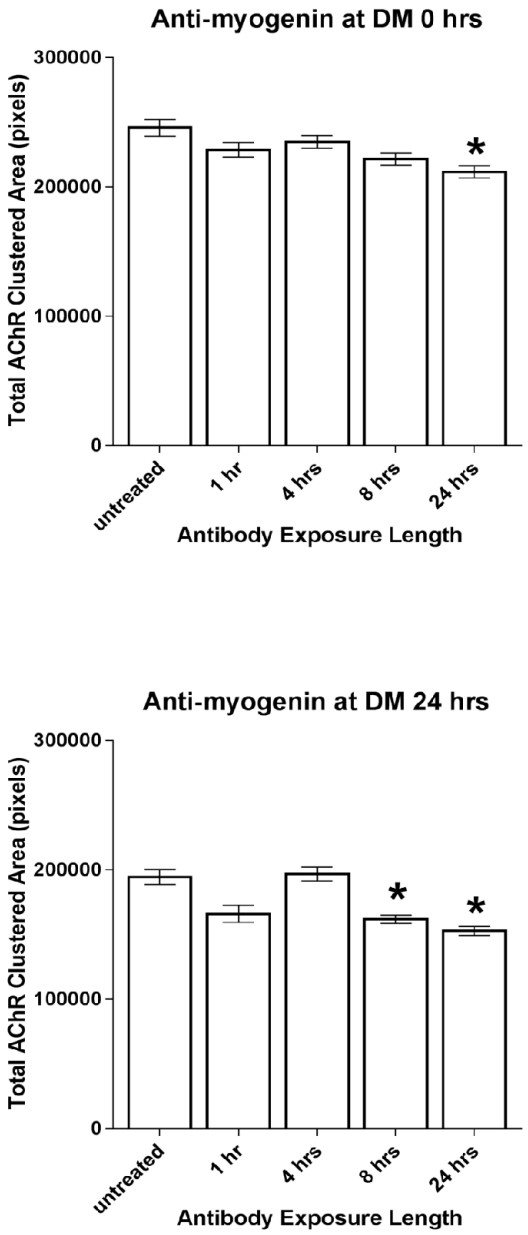

Figure 6: 1 µg/ml antibody to myogenin for as few as 8 hours can decrease the frequency of agrin-induced AChR clusters. Clustering was quantified by analyzing fluorescent images from cultures that were untreated or exposed to 1 µg/ml antibody for variable time periods beginning with the introduction of DM (A), or after 24 hours in DM (B). Cultures were fixed and images captured at 72 hours in DM. The histograms reveal that exposure to 1 µg/ml antibody to myogenin for as few as 8 hours in DM, beginning after 24 hours in DM, was sufficient to decrease agrin-induced AChR clusters relative to untreated cultures or cultures treated for fewer hours, using Student's t-test at $p<0.05$. For cultures exposed to antibody to myogenin beginning with the introduction of DM, 1 µg/ml antibody for 8 hours or less was insufficient to have an effect. (*) statistically decreased from untreated cultures. Error bars indicate standard error of the mean (s.e.m.).

transcription of genes within this open chromatin state, including activating genes involved in neuromuscular synapse formation, and genes that shut down the cell proliferation machinery. This leads to myoblast differentiation and exit from the cell cycle, and the fusion of myoblasts into multinucleated myotubes [47]. Indeed, ectopic expression of myogenin in proliferating myoblasts is sufficient to activate a collection of genes that mediates exit from the cell cycle [48].

In development TATA binding protein associated factors (TAFs) interact with TATA binding protein related factors (TRFs) to activate tissue specific transcription. TAFs and TRFs are subunits that combine as core promoter factors specific to various tissues. In skeletal muscle the combination of TAF3 and TRF3 (TAF3/TRF3) drives the differentiation of myoblasts into myotubes [49,50]. First, the TAF3/TRF3-dependent transcriptional mechanism results in a high level of MyoD expression [49,51]. Then MyoD targets TAF3/TRF3 to activate myogenin expression and downregulate Myf5 expression [52]. This switch from Myf5 to myogenin coincides with cell cycle exit and commitment to differentiate [48]. Myogenin then activates additional genes for myotube differentiation [7].

Previously it was demonstrated that continuous exposure over several days to a lower level of antibody, 1 µg/ml antibody to MyoD or myogenin, decreased agrin-induced AChR clustering [8]. The current data show that this concentration of antibody to myogenin, or exposure to myogenin morpholino, decreased agrin-induced AChR clustering with shorter exposure times if exposure was early in myotube formation. With a higher concentration, 5 µg/ml antibody to MyoD or myogenin, exposure anytime during myotube formation decreased agrin-induced AChR clustering. The current data suggest that the C2C12 cell culture system is more sensitive to antibody to myogenin than antibody to MyoD, and more sensitive to antibody to myogenin or myogenin morpholino when exposed early in myotube formation when myoblasts are first fusing into myotubes.

MyoD targets myogenin gene expression, and also targets MuSK and rapsyn gene expression. Myogenin targets rapsyn but not MuSK gene expression [7], and also targets genes for AChR subunits [26,27]. Therefore, MyoD has a role earlier in differentiation involving MuSK and rapsyn, while myogenin has a role later in differentiation involving rapsyn and AChR but not MuSK. The results in the present study establish more specifically how MyoD and myogenin interact in neuromuscular synapse formation by demonstrating that exposure to 5 µg/ml antibody to MyoD reduced myogenin gene expression concurrent with a decrease in agrin-induced AChR clustering. This

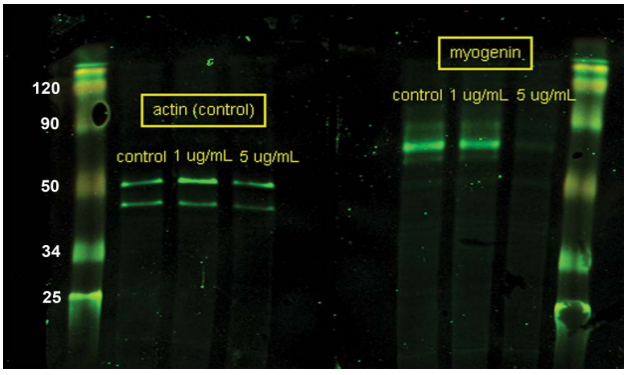

Figure 7: 5 µg/ml antibody to MyoD decreased myogenin gene expression. Untreated agrin-induced cultures were compared with cultures exposed to 1 or 5 µg/ml antibody to MyoD for 24 hours beginning after 48 hours in DM. 5 µg/ml antibody to MyoD decreased myogenin gene expression but 1 µg/ml was insufficient to do so. Cultures were assayed for the amount of myogenin protein and compared with an actin control using Western Blots.

suggests that MyoD is essential for agrin-induced AChR clustering through a mechanism that includes activation of myogenin gene expression, leading to activation of AChR gene expression, and ultimately production of an appropriate level of AChR for agrin-induced AChR clustering and neuromuscular synapse formation.

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