Monoclonal Antibodies Against Muscleblind-like 3, a Protein with Punctate Nuclear Localization

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Muscleblind-like 3 (MBNL3) belongs to a family of RNA binding proteins that regulate alternative splicing. We have generated a set of monoclonal antibodies (MAbs) against mouse MBNL3, three of which do not cross-react with the other muscleblind-like (MBNL) proteins, MBNL1 and MBNL2. Epitope mapping revealed that MAbs P1C7, P1E7, SP1C2, and P2E6 recognize distinct, non-overlapping segments of the MBNL3 polypeptide sequence. Immunohistochemical staining of proliferating muscle precursor cells localized MBNL3 to the nucleus in a punctate pattern, characteristic of subcellular structures in the nucleus enriched in pre-messenger RNA splicing factors. Although MBNL3 did not co-localize with SC35 and PSP1 (widely used markers of splicing speckles and paraspeckles), the punctate localization pattern of MBNL3 within interchromatin regions of the nucleus is highly predictive of proteins involved in pre-mRNA processing. Monoclonal antibodies specific for mouse MBNL3 will facilitate further investigation of the expression pattern and unique functions of this splicing factor during development and in different adult mouse tissues.

Introduction

THE MUSCLEBLIND-LIKE (MBNL) FAMILY of proteins spreads across the phylogenetic tree from *Drosophila* to humans. The *Drosophila* genome contains one muscleblind (*mbi*) gene, while in vertebrates, MBNL proteins are encoded by three genes: *Mbnl1*, *Mbnl2*, and *Mbnl3*.⁽¹⁾ MBNL proteins are regulators of alternative splicing that directly bind pre-mRNA.⁽²⁾ The RNA binding activity of MBNL1 requires the conserved Cys3His zinc finger domains, a feature found in many proteins involved in RNA processing.⁽³⁾

The mammalian MBNL proteins are thought to be functionally similar based on their high degree of sequence homology. Studies examining MBNL transcript levels have revealed distinct patterns of expression for MBNL1, MBNL2, and MBNL3 during development and in the adult mouse.⁽⁴⁻⁶⁾ These findings suggest that MBNL1, MBNL2, and MBNL3 may display distinct cellular functions in different tissues. Knowledge about protein expression patterns in the adult animal can provide insights into a protein's physiological function. Therefore, we set out to generate monoclonal antibodies that selectively recognize MBNL3. The development of such reagents will open many avenues for investigating the function of MBNL3 in vertebrates.

Material and Methods

Bacterial expression plasmids

Mouse MBNL3 coding sequence as a HindIII-BamHI fragment was inserted into pET28c vector for expression of His-tagged mouse MBNL3 in bacteria. An expression plasmid for His-tagged human MBNL3 was constructed by cloning the human MBNL3 cDNA as an EcoRI fragment into the EcoRI site of pET28a. Insert orientation was determined by restriction enzyme digestion. Mouse MBNL1 coding sequence, as a BamHI to EcoRI fragment, and human MBNL1 coding sequence, as a BamHI to XhoI fragment, were subcloned into pET28b for the expression of these proteins with a His-tag in bacteria. cDNAs for mouse and human MBNL2 were PCR amplified with EcoRI and HindIII cohesive ends and subcloned into pET28b vector to construct expression plasmids for N-terminal His-tagged fusions. Fragments encoding mouse MBNL3 deletion mutants as BamHI-NotI fragments were subcloned into pET28b for expression as His-tagged proteins or as EcoRI-NotI fragments into pGEX-4T-2 vector for GST-fusions. C-terminal fragments of mouse MBNL3 were PCR amplified with BamHI and EcoRI cohesive ends and cloned into pGEX-KT vector for expression as GST fusions. The coding sequence of all bacterial expression constructs was confirmed by DNA sequencing.

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Expression and purification of recombinant MBNL proteins

Bacterial strain BL21 was transformed with the appropriate expression vector and grown in LB containing 100 µg/mL ampicillin (GST fusion) or 30 µg/mL kanamycin (His-tag protein). Overnight cultures were diluted 1:20 into 100 mL LB with the appropriate antibiotic. Cells were grown at 37°C to OD₆₀₀ of 1.0. Fusion protein expression was induced with the addition of IPTG to 0.1 mM final concentration and cultures were grown for 16–18 h at 17°C. For Western blot analysis, bacteria were pelleted, lysed by sonication in 25 mM Tris-HCl (pH 7.9), 0.5 M NaCl, 10% glycerol, and 1 mM DTT with protease inhibitors, and subjected to centrifugation to remove insoluble material. For the purification of proteins, bacterial pellets were sonicated in 0.4 M HEMG buffer (25 mM HEPES [pH 7.9], 0.1 mM EDTA, 12.5 mM MgCl₂, 0.4 M KCl, 10% glycerol, 0.1% NP-40) containing protease inhibitors and 1 mM DTT. For GST-fusion proteins, lysates clarified by centrifugation were incubated with glutathione-sepharose 4B (GE Healthcare, Piscataway, NJ) for 2 h at 4°C. After extensive washing, bound GST-fusion proteins were eluted with 20 mM glutathione into 0.4 M HEMG (adjusted to pH 8.0). Induction and purification of His-tagged proteins were carried out essentially as described above with the following modifications. One mM DTT was omitted from all buffers and replaced with 5 mM β-mercaptoethanol. His-tagged proteins were purified on Ni-NTA agarose (Qiagen, Valencia, CA) and eluted with 200 mM imidazole. Purified proteins were subjected to SDS-polyacrylamide gel electrophoresis and visualized by Coomassie blue staining to assess protein yield and purity.

Production of hybridoma cultures

Two Robertsonian mice (RBF/DnJ) were injected with ~100 µg of purified MBNL3 protein emulsified in RIBI adjuvant. After 3 weeks, immunized animals were boosted with ~100 µg MBNL3 protein without adjuvant followed by a second injection 7–10 days later. Ten days after the last boost, test bleeds were taken and the antisera tested for the presence of anti-MBNL3 antibodies by Western blot analysis. A strong positive signal was required before proceeding to the cell fusion stage. Two mice with the strongest immune response were given a final injection exactly 3 days before fusion. The spleen was harvested and fused to FOX-NY myeloma cell line as described.⁽⁷⁾

Screening of hybridoma cells

Supernatants of hybridoma cultures were screened for production of anti-MBNL3 antibody by ELISA. 96-well plates were coated with purified GST-MBNL3 protein. Hybridoma supernatants (diluted 1:10) were added to each well and incubated for 1 h at room temperature then washed with 1× PBS. HRP-conjugated anti-IgG (1:2000 dilution) was added and incubated at room temperature for 30 min. After washing and addition of HRP substrate ABTS, plates were incubated for 10–15 min at room temperature and read at OD₄₀₅. Positive supernatants were tested in Western blots using purified recombinant GST-MBNL3 separated on SDS-polyacrylamide and transferred to nitrocellulose. Hybridoma supernatants, as primary antibody, were diluted 1:2 and incubated overnight at 4°C. Positive cultures were chosen for clonal expansion.

Cloning of positive monoclonal cell lines

Positive hybridoma culture wells were cloned as follows. Cells were diluted to 1×10⁴/mL in RPMI media. Next, 100 µL of this cell suspension were further diluted to 10 mL in RPMI 1X AAT supplemented with 20% FBS and plated at 100 µL/well in a 96-well plate at 1 cell/well. Cells were expanded and the secretion of anti-MBNL3 antibody was monitored by Western blotting. Fusion plates were frozen by aspirating the supernatant and adding 100–200 µL of freezing media (90% FBS and 10% DMSO) directly to each well. The plates were stored at –80°C and kept up to 1 year.

Isotyping of monoclonal antibodies

Hybridoma culture supernatants were isotyped using a mouse monoclonal antibody isotyping kit (Roche, Indianapolis, IN) or capture ELISA and reaction with HRP-conjugated specific secondary antibodies (Southern Biotech, Birmingham, AL). When using the Roche isostrips, culture supernatants were diluted 1:100 to 1:1000.

Cell culture and whole cell lysates

C2C12 mouse myoblast cell line was maintained in Dulbecco modified Eagle's medium (DMEM) with 10% FBS, penicillin/streptomycin, and 2 mM L-glutamine in a humidified incubator at 37°C with 5% CO₂. Differentiation of C2C12 myoblasts into myotubes was achieved by culturing cells, which had reached 70–80% confluency, in DMEM containing 2% horse serum, penicillin/streptomycin, L-glutamine, and ITS liquid supplement (10 ng/mL insulin, 5.5 µg/mL transferrin, and 10 ng/mL selenium). Cells were maintained in differentiation media for up to 4 days, with media changes every 2 days. EL4 mouse cells were maintained in RPMI media with 10% FBS, penicillin/streptomycin, and 2 mM L-glutamine in a humidified incubator at 37°C with 5% CO₂. Whole cell lysates were prepared as follows: EL4 cells were washed twice with PBS, resuspended in cell lysis buffer (50 mM Tris-HCl [pH 7.4], 400 mM NaCl, 1% NP-40, 1 mM DTT, 1 mM PMSF, 1 µg/mL aprotinin, 1 µg/mL leupeptin, 1 µg/mL pepstatin) and lysed by sonication. Whole cell extracts were collected after centrifugation at 4°C to remove insoluble material.

Western blot analysis

Purified recombinant proteins were loaded onto SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and probed with the following primary antibodies at the indicated dilutions: 1:200–500 MAb P1C7; 1:200–500 MAb P1E7; 1:100 MAb SP1C2; 1:20 MAb P2E6; 1:1000 MBNL1 MAb Mb1a (kindly provided by G.E. Morris), 1:1000 anti-GST polyclonal antibody, 1:2000 rabbit anti-6× His polyclonal (Bethyl Laboratories, Montgomery, TX). The appropriate horseradish peroxidase-conjugated secondary antibody was used at 1:5000 dilution. Proteins of interest were detected by chemiluminescence (Pierce/Thermo Scientific, Rockford, IL).

Immunocytochemistry

Cells were fixed with 2% paraformaldehyde, permeabilized with 0.5% Triton X-100, and blocked with 1% normal goat serum solution in PBS. Primary and secondary antibodies were diluted in 1% goat serum-PBS solution. The

primary antibodies against mouse MBNL3 (MAb P1E7) and against MBNL1 (MAb Mb1a) were used at dilutions of 1:3. Anti-SC35 IgG1 monoclonal antibody (Abcam, Cambridge, MA) was used at 1:1000 dilution, anti-PSP1 rabbit polyclonal antibody (Dundee Cell Products, Dundee, Scotland) at 1:2000 dilution, and anti-MBNL3 IgG2b MAb P2E6 undiluted. Primary antibody reactions were incubated for 1 h at room temperature or overnight at 4°C. Fluorescein-conjugated secondary antibodies were diluted 1:1000 and applied for 2 h at room temperature. Goat anti-mouse IgG1-Texas Red, goat anti-mouse IgG2-FITC (Southern Biotech), and goat anti-rabbit-Texas Red secondary antibodies were used for colocalization experiments. DAPI staining was used to visualize DNA in nuclei. Samples were mounted with Vectashield and visualized using a Nikon Eclipse E600 microscope. Two different filters (UV-2A and FITC-HYQ) were employed to capture the stained images.

Results

Isolation of mouse MBNL3 monoclonal antibodies

Monoclonal antibodies against the mouse MBNL3 protein were generated by immunizing mice with affinity purified N-terminal histidine-tagged mouse MBNL3. After four

injections over a 2-month period, the amount of anti-MBNL3 antibody in the sera of immunized animals was determined by Western blot analysis. The hybridoma method was carried out using the spleen of the two mice that mounted the strongest immune response against MBNL3. We screened hybridoma cultures for the production of anti-MBNL3 antibodies by ELISA and Western blot using recombinant mouse MBNL3 purified as a GST fusion. Clonal expansion of positive cultures resulted in the establishment of four hybridoma cell lines (P1C7, P1E7, P2E6, SP1C2) that produced monoclonal antibodies recognizing recombinant mouse MBNL3 (Fig. 1A). The isotype of the antibodies produced by hybridomas P1C7, P1E7, and SP1C2 was determined to be IgG1 while P2E6 produced IgG2b antibodies.

Endogenous proteins detected by each monoclonal antibody was examined using whole cell lysates prepared from EL4 cells, a mouse cell line that we found expressed higher levels of MBNL3 transcript than C2C12 cells (Fig. 1C). The predominant protein detected by all four monoclonal antibodies in EL4 T cell lysates migrated around 40 kDa, the expected size for MBNL3 (Fig. 1D). In a longer exposure, MABs P1E7 and P2E6 also detected a smaller protein around 34 kDa in size (Fig. 1D, right panel). Two alternatively spliced transcripts for MBNL3 have been annotated, each of which lacks

FIG. 1. Mouse monoclonal antibodies for mouse MBNL3 protein. (A, B) The indicated mouse (m) or human (h) MBNL1 (1), MBNL2 (2), or MBNL3 (3) proteins expressed with N-terminal 6 $\times$ His-tag were resolved on 10% SDS-polyacrylamide and subjected to Western blotting with anti-His antibody or the indicated monoclonal antibody. Proteins recognized by each antibody were detected by chemiluminescence. The positions of molecular weight standards are shown at left. (C) Total RNA was isolated from the indicated mouse cell lines. MBNL3 transcript levels were determined by qRT-PCR. Rplp0 mRNA levels were used to control for RNA input. Transcript levels relative to the amount in C2C12 cells were calculated using the conventional $2^{-\Delta\Delta C_t}$ method. (D) The indicated mouse monoclonal antibodies were incubated with whole cell lysates from EL4 mouse cells (100 μ g) immobilized on nitrocellulose for Western blot analysis. Antibody bound proteins were visualized by chemiluminescence. Right panel shows a longer exposure of the indicated lanes. Arrow indicates the alternatively spliced truncated isoform of MBNL3. The migration of molecular weight standards is shown on the left. Anti-MBNL3 monoclonal antibodies: P1C7, P1E7, P2E6, SP1C2, anti-MBNL1 MAb: Mb1a.

the translation start site for full-length MBNL3 but retains a downstream ATG start codon in frame. The predicted size of the truncated translation product is ~35 kDa, which corresponds to the faster migrating band detected by MAb P1E7 and P2E6 in Western blots.

Characterization of mouse MBNL3 monoclonal antibodies

MBNL3 displays a high degree of amino acid identity to the muscleblind-like proteins MBNL1 and MBNL2, in particular within their Cys3His zinc finger domains.⁽⁸⁾ The specificity of the four MAb—P1C7, P1E7, P2E6, and SP1C2—for MBNL3 was examined by Western blot analysis using recombinant mouse and human MBNL1, MBNL2, and MBNL3 (Fig. 1A, B). We observed that mouse MBNL3 was efficiently recognized

by all four monoclonal antibodies (Fig. 1A, lanes m3). MAb P1C7 and SP1C2 also recognized recombinant human MBNL3 whereas P1E7 and P2E6 did not (Fig. 1B, lanes h3). MAb P1C7 further distinguished itself from the other three monoclonal antibodies by cross-reacting with the human and mouse MBNL1 and MBNL2 proteins (Fig. 1A, B). These data led us to conclude that the epitope for MAb P1C7 is unique from those for P1E7, P2E6, and SP1C2 and consists of amino acids conserved in all three MBNL proteins expressed in mice and humans.

Epitope mapping of MBNL3 MAb

As the first step towards mapping the epitope for each monoclonal antibody, we expressed and purified a set of MBNL3 C-terminal deletion mutants as N-terminal 6xHis- or GST-fusion proteins (Fig. 2A, B). The epitopes for MAb P1E7

FIG. 2. Epitope mapping of mouse MBNL3 MAb. (A) Schematic of mouse MBNL3 deletion mutants. The positions of Cys3His (C3H) zinc finger motifs and MBNL3 deletion fragments are shown. The N-terminal tag and terminating amino acid for each deletion is provided. (B) MBNL3 C-terminal deletion mutants expressed in bacteria with the indicated N-terminal tag were subjected to SDS-PAGE and stained with Coomassie blue. Asterisks indicate the position of the intact deletion mutants. (C) Proteins shown in B were transferred to nitrocellulose and the membranes incubated with the indicated anti-MBNL3 monoclonal antibody. Proteins bound by antibody were visualized by chemiluminescence. Figures at left indicate the relative positions of molecular weight standards.

and P2E6 were found to reside within the last 100 amino acids of mouse MBNL3, as a positive signal was not detected when this portion of the protein was removed (Fig. 2C, His241). These findings are consistent with our earlier observation that MAbs P1E7 and P2E6 detected an N-terminal truncated isoform of MBNL3 present in EL4 cells. The C-terminus of the MBNL proteins is the least conserved and displays little sequence homology among family members, making it the likely region for antibody epitopes unique to mouse MBNL3. By contrast, MAb P1C7 and SP1C2 detected all deletion mutants except the shortest fragment, which contained only the first 52 amino acids of mouse MBNL3 (Fig. 2C, GST52). These data map the epitopes for MAbs P1C7 and SP1C2 between amino acids 52 and 114 of mouse MBNL3 and those for MAbs P1E7 and P2E6 between amino acids 241–342.

The discovery that P1C7 but not SP1C2 cross-reacted with mouse and human MBNL1 and MBNL2 allowed us to more precisely delineate the epitopes for these two monoclonal antibodies. Alignment of the MBNL proteins from the two species revealed that between amino acids 52–114, amino acids 53–92 are nearly 100% identical in all six proteins, suggesting that the epitope for MAb P1C7 most likely maps to this region (Fig. 3, underscored in purple). By contrast, MAb SP1C2 does not recognize MBNL1 or MBNL2 and only de-

tected mouse and human MBNL3. Therefore the epitope for SP1C2 should be conserved in mouse and human MBNL3 but not in the MBNL1 and MBNL2 sequences. A segment of mouse MBNL3 between residues 92–114 that fulfills these criteria can be found between amino acids 104–114 and can be extended to amino acid 119 (Fig. 3, underscored in green).

To more precisely map the epitopes for MAbs P1E7 and P2E6, bacterial expression vectors for three overlapping fragments containing the C-terminal 84 (CT84), 57 (CT57), and 30 (CT30) amino acids of mouse MBNL3 fused to GST were constructed (Fig. 4A). When the purified proteins were examined in Western blots with full-length His-tagged MBNL3, we observed that both P1E7 and P2E6 detected MBNL3 fragments CT84 and CT57 but not CT30 (Fig. 4B). These results suggest that the epitopes for P1E7 and P2E6 lie between amino acids 286–315 of mouse MBNL3 (Fig. 3, underscored in black). Interestingly, the detection of an additional lower molecular weight, breakdown product of CT84 by MAb P2E6 but not by MAb P1E7 or anti-GST antibody suggests that the epitope for P2E6 differs from and is C-terminal of the P1E7 recognition sequence. In summary, we have established four independent hybridoma cell lines that produce MBNL3 monoclonal antibodies that recognize distinct, non-overlapping segments of the mouse MBNL3 polypeptide.

FIG. 3. MBNL3 MAbs recognize non-overlapping epitopes in mouse MBNL3. Sequence alignment of MBNL1, MBNL2, and MBNL3 proteins from mouse (m) and human (h) is shown. Residues 100% identical in all six sequences are shown in red. Conservation >50% is indicated in blue. The putative location of epitopes for MAb P1C7 (purple), SP1C2 (green), and P1E7/P2E6 (black) is delineated by the colored bars underscoring the sequence alignment.

FIG. 4. Fine mapping the epitopes of MAb P1E7 and P2E6 in mouse MBNL3. **(A)** Schematic of mouse MBNL3 protein fragments. Positions of Cys3His (C3H) zinc finger domains are indicated. The starting amino acid and location of C-terminal MBNL3 fragments (CT84, CT57, CT30) are provided. **(B)** His-tagged full-length MBNL3 (WT) and GST-tagged C-terminal fragments were separated on SDS-polyacrylamide and transferred to nitrocellulose. Immobilized proteins were subjected to Western blotting using the indicated MBNL3 MAb or anti-GST polyclonal sera and detected by chemiluminescence. The positions of molecular weight standards are shown at left.

Localization of MBNL3 to nuclear punctate pattern in proliferating mouse myoblasts

We have reported that MBNL3 protein is expressed in proliferating mouse myoblasts and that MBNL3 protein levels declined as the cells differentiated into myotubes.⁽⁸⁾ The expression level of MBNL1 remains unchanged during muscle differentiation.^(6,9) However, MBNL1 rapidly redistributes from the nucleus to the cytoplasm in cultures of primary human myoblasts executing the muscle differentiation program.⁽⁹⁾ These results prompted us to examine the distribution and localization of MBNL3 in myogenic cells.

In proliferating C2C12 mouse myoblasts, MBNL3 was repeatedly detected in the nuclei of cells in a punctate pattern (Fig. 5A). The intensity of the punctate staining decreased after the cells were cultured for 16 h in differentiation media (Fig. 5B). The signal for MBNL3 was undetectable after 24 h and remained absent after 4 days of differentiation, when multinucleated myotubes were clearly visible (Fig. 5C, D, and data not shown). Similar analysis using Mb1a, a monoclonal antibody raised against human MBNL1,⁽⁹⁾ revealed a very different staining pattern. MBNL1 was found to be more prominent in the nucleus but was still detectable above background levels in the cytoplasm of proliferating C2C12 cells (Fig. 5I). In contrast to MBNL3, the nuclear distribution of MBNL1 was uniform, with the protein clearly excluded from vesicles that resembled nucleoli. No change in the protein levels or nuclear to cytoplasmic distribution of MBNL1 was observed as C2C12 cells progressed from proliferating myoblasts to terminally differentiated myotubes (Fig. 5I–L). These studies indicate that in C2C12 mouse myoblasts the cellular localization, expression pattern, and protein levels

FIG. 5. MBNL3 and MBNL1 expression differs in proliferating and differentiating C2C12 myoblasts. C2C12 myoblasts were maintained in growth media (GM) or differentiation media (DM) for the indicated time. Expression levels and localization of MBNL3 and MBNL1 were determined by immunocytochemical staining using MAbs P1E7 and Mb1a, respectively. Nuclei were visualized by DAPI staining (blue). Scale bars, 50 μ m.

of MBNL3 and MBNL1 differ dramatically during muscle differentiation.

Proteins involved in pre-messenger RNA processing often are localized to nuclear speckles, distinct subnuclear structures marked by small nuclear ribonucleoproteins^(10,11) and the splicing factors SC35^(9,12) and 9G8.⁽¹³⁾ Nuclear speckles are primarily storage, modification, and assembly sites for pre-mRNA processing proteins.⁽¹⁴⁾ Using a mouse monoclonal against SC35 with an isotype different from MAb P2E6, we detected no overlap of the staining patterns of MBNL3 and SC35 in the nuclei of proliferating C2C12 myoblasts (Fig. 6A–C). Nuclei contain additional structures that display a speckled pattern of staining, including paraspeckles, a class of subnuclear bodies that form around long non-coding RNAs and contain paraspeckle protein 1, PSP1.^(15,16) Co-staining for MBNL3 and PSP1 revealed no co-localization of these two proteins in the nuclei of proliferating mouse myoblasts (Fig. 6D–F). One feature that MBNL3 did have in common with SC35 and PSP1 was that all three proteins were located in the interchromatin regions of the nucleus, which contain little or no DNA as judged by DAPI staining (Fig. 6A, B, E). The subnuclear localization pattern of MBNL3 in interchromatin regions supports our discovery that MBNL3 is a regulator of pre-mRNA splicing in myogenic cells.⁽¹⁷⁾

Discussion

Mammalian MBNL proteins have been implicated in muscle differentiation based on their homology to mu-

sclblind, a *Drosophila* protein required for terminal muscle differentiation in flies.⁽¹⁸⁾ The existence of three MBNL genes in vertebrates as opposed to a single gene in *Drosophila* implies that there are functional differences between the three mammalian MBNL proteins MBNL1, MBNL2, and MBNL3. While MBNL1 is thought to promote muscle differentiation, we found that MBNL3 acts in an opposing manner and inhibits muscle differentiation.^(8,19) Monoclonal and polyclonal antibodies against MBNL1 revealed that MBNL1 expression levels do not decrease during muscle differentiation, a pattern that has been observed for MBNL2 and MBNL3.^(6,9) We have generated monoclonal antibodies against mouse MBNL3 by immunizing animals with the full-length mouse protein and found that MBNL3 is expressed in proliferating muscle precursor cells and not in terminally differentiated myotubes.

Monoclonal antibodies against all three human MBNL proteins have been described and used to characterize the expression pattern of these proteins in human cells.⁽⁹⁾ Holt and colleagues⁽⁹⁾ reported that they were unable to detect MBNL3 in primary human myoblasts by Western blot analysis or immunocytochemistry using the human Mbnl3 MAb Mb3a. Using the same immunological reagent, we detected endogenous MBNL3 at very low levels in proliferating C2C12 mouse myoblasts. Our estimation is that MBNL3 is at least 10 times less abundant than MBNL1 at both the mRNA and protein levels in proliferating mouse myoblasts. These findings suggest that MBNL1 and MBNL3 have distinct roles during muscle development in the embryo and in muscle regeneration in the adult. In addition, we observed that the

FIG. 6. MBNL3 does not co-localize with SC35- or PSP1-marked nuclear speckles in proliferating C2C12 mouse myoblasts. C2C12 cells maintained in growth media were fixed, permeabilized, and subjected to immunocytochemistry. (A, D) Staining with MBNL3 MAb P2E6, isotype IgG2b (green). DAPI staining for visualizing chromatin and nuclei (blue). (B) Staining with SC35 MAb, isotype IgG1 (red). DAPI staining shown in blue. (C) Merging of MBNL3, SC35, and DAPI images. (E) Staining with PSP1 rabbit polyclonal antibody (red); DAPI staining (blue). (F) Merging of MBNL3, PSP1, and DAPI images. Scale bars, 10 μ m.

cellular distribution of MBNL1 in mouse myoblasts during muscle differentiation differed from what has been reported for human primary myoblasts. Therefore, species differences between MBNL orthologs may exist and could explain why it has been difficult to recapitulate all aspects of the human disease, myotonic dystrophy, in mouse models.

The nucleus is highly compartmentalized with protein factors localized to distinct structures. These structures include nuclear speckles, paraspeckles, YT-521B structures, Cajal bodies, and "gems," all of which demonstrate a speckled staining pattern when visualized by indirect immunocytochemistry.⁽¹⁴⁾ Localization to the nucleus in a speckled pattern is a feature of many proteins involved in pre-mRNA processing. Nuclear speckles are thought to represent the primary site of modification and assembly of proteins involved in pre-mRNA splicing. Although MBNL3 did not co-localize with the splicing factor SC35 found in nuclear speckles or with PSP1, a marker of paraspeckles, the punctate nuclear localization pattern of MBNL3 within interchromatin regions is highly diagnostic of proteins involved in pre-mRNA splicing, further suggesting that MBNL3 functions as an alternative splicing factor in vertebrates.

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Author Disclosure Statement

The authors have no financial conflicts to disclose.

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