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Expression Pattern of Muscleblind-like Proteins Differs in Differentiating Myoblasts

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Abstract

Muscleblind-like (MBNL) proteins are believed to be regulators of myogenesis and are implicated in myotonic dystrophy. While *Drosophila melanogaster* muscleblind is required for terminal muscle differentiation, mammalian MBNL3 functions as an inhibitor of myogenesis. In this study, we analyzed the expression pattern of MBNL3 in different adult mouse tissues and tissue culture cells. MBNL3 transcript is enriched in the lung, spleen, and testis and not in heart and skeletal muscle. By western blotting, we found that MBNL3 was expressed in C2C12 myoblasts and ts13 myofibroblasts, but was detected at significantly lower levels in fibroblasts. MBNL3 protein levels decreased when cells were shifted to muscle differentiation conditions, but the closely related MBNL1 protein was unaffected. These results suggest that myoblasts and fibroblasts respond to differentiation conditions by activating signaling pathways that repress MBNL3 but not MBNL1 expression.

Keywords

Muscleblind; MBNL proteins; muscle differentiation; gene expression; myoblasts; fibroblasts; Cys3His zinc finger

Introduction

Drosophila melanogaster Muscleblind (Mbl) is a nuclear, Cys3His zinc finger protein expressed late in larval development and required for terminal differentiation of muscle and photoreceptor cells [1,2]. The Muscleblind family of proteins spreads across the phylogenetic tree from *Drosophila* to humans [3]. Mammalian homologues, known as the muscleblind-like (MBNL) proteins, contribute to the pathogenesis of the neuromuscular degenerative disease, myotonic dystrophy (DM) [4–7].

Myotonic dystrophy is an autosomal dominant disorder caused by CTG expansions in the 3'-UTR of the myotonic dystrophy protein kinase (*DMPK*) gene (type 1, DM1) [8–10] or CCTG expansions in the first intron of *Znf9* (type 2, DM2) [11]. Common to DM1 and DM2 is the expression of expanded mutant RNA transcripts, which accumulate in the nuclei of diseased cells [12–14]. This observation has led to the RNA dominant model, which proposes that the mutant RNAs sequester and hinder the activity of RNA-binding proteins crucial for proper muscle development and function [15,16]. The isolation of proteins that bind to expanded CUG

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repeats led to the identification of EXP42, later renamed MBNL1 and founder of the MBNL protein family [17].

The three mammalian MBNL proteins, MBNL1, MBNL2 and MBNL3, contain four highly conserved Cys3His zinc finger motifs [4,18]. MBNL3 exhibits 43% amino acid identity to Mbl, and nearly 60% identity to MBNL1 [18]. There is evidence that the different MBNL/Mbl proteins may be functionally distinct. While Mbl is involved in late stage muscle differentiation in flies, MBNL3 functions in an opposing manner in mammalian cells. Overexpression of MBNL3 in C2C12 mouse myoblasts inhibits the induction of terminal muscle differentiation markers, reduces MyoD dependent gene transcription and hinders myotube formation [18, 19]. Loss of MBNL3 function accelerated the process of muscle differentiation. These data suggest that MBNL3 is an inhibitor of muscle differentiation. The precise function of MBNL1 and MBNL2 during myogenesis remains to be determined.

In this study, we examined the expression pattern of MBNL3 in different adult mouse tissues and mammalian tissue culture cells. MBNL3 is not highly expressed in cardiac or skeletal muscle, consistent with its function as an inhibitor of muscle differentiation. A comparison of MBNL3 and MBNL1 expression patterns revealed some striking differences. MBNL3 expression decreased when C2C12 myoblasts were induced to differentiate. By contrast, the expression of MBNL1 was unaltered and did not appear to be subject to myogenic regulation. These data suggest that the MBNL proteins may have unique functions during muscle differentiation.

Materials and methods

Cell culture

Mouse myoblasts (C2C12), fibroblasts (10T1/2 and NIH3T3) and hamster myofibroblasts (ts13) were cultured in DMEM with 10% FBS, 1% penicillin/streptomycin, 2 mM L-glutamine. C2C12 and 10T1/2 cells were maintained at 37° C in a humidified incubator containing 5% CO₂. ts13 cells were propagated at 33.5° C. Differentiation of C2C12 myoblasts was achieved by switching cells at 70–80% confluency from DMEM + 10%FBS to DMEM containing 2% horse serum, penicillin/streptomycin, L-glutamine, and ITS (10 ng/ml insulin, 5.5 µg/ml transferrin and 10 ng/ml selenium) supplement. Cells were maintained in differentiation media for up to 5 days, with media changes every 2 days.

RNA extraction and real-time PCR

Total RNA was purified using the RNeasy Mini Kit (QIAGEN) and the yield determined by measuring OD₂₆₀. RNA samples were subjected to real-time PCR using Brilliant SYBR Green QPCR Master Mix (Stratagene) in 10 µl final volume that included the following: 25 ng total RNA, 10 nM each of forward and reverse primers, reverse transcriptase (Stratagene, #600085), reference dye, and 2X SYBR Green Master mix. Reactions were processed using Stratagene MX3000P system with 40 cycles of 95° C for 30 seconds, 55° C for 60 seconds, 72° C for 30 seconds after a 30 minute reverse transcription reaction at 45° C. Primers for MBNL1 are 5'-CCCTCCACCGCACTTAAAGTC-3' (forward) and 5'-GCCAGGATGAGTCATGTAAGGAT-3' (reverse). Primers for MBNL3 are 5'-CTCATGCTGCAGAACGCTCA-3' (forward) and 5'-GAACTCCTGGCAGTGCAAGA-3' (reverse). As the internal control, primers against acidic ribosomal phosphoprotein P0 (ARPP) were used: 5'-TGTTTGACAACGGCAGCATTT-3' (forward), 5'-CCGAGGCAACAGTTGGGTA-3' (reverse). ΔCt for each gene was calculated and represents the difference between the Ct value for the gene of interest and the Ct value for ARPP. Fold changes were calculated using the 2^{-ΔΔCt} convention.

Preparation of whole cell extracts and western blotting

Cells were washed twice with phosphate buffered saline, resuspended in lysis buffer (50mM Tri-HCl, pH 7.5, 400 mM NaCl, 1% NP-40, 1 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 1 mM DTT), and incubated on ice for 20 minutes with vigorous vortexing for 15 seconds every 5 minutes. Supernatants were collected after centrifugation at 14,000 rpm for 10 min. Protein concentration of lysates was determined by Bio-Rad Protein Assay. For immunoblotting, approximately ~100 µg protein was subjected to SDS-PAGE, transferred onto nitrocellulose, and probed with one or more of the following primary antibodies: 1:500 dilution anti-MBNL3 polyclonal serum; 1:1000 dilution anti-MBNL1 mAb clone HL 1822(3A4-1E9) (Sigma), 1:1000 dilution anti-TBP polyclonal antibody, 1:5000 dilution anti-alpha-tubulin (Abcam), and 1:1000 dilution GAPDH mAb 6C5 (Adv. ImmunoChem Inc.). The appropriate horseradish peroxidase-conjugated secondary antibodies were used at 1:5000 dilution. Signals were visualized using an ECL detection kit (GE Amersham Biosciences).

Preparation of cytosolic and nuclear extracts

Cell pellets were resuspended gently in 5 packed cell volumes (PCV) of hypotonic buffer A (10 mM HEPES pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 1 µg/ml leupeptin, 2 µg/ml aprotinin, 1 µg/ml pepstatin A, 0.5 mM PMSF) and incubated on ice for 15 minutes. Cell suspension was centrifuged for 5 minutes at 420 x g and the supernatant discarded. Intact pelleted cells were resuspended in 1 PCV of buffer A and pushed through a 26 gauge needle to disrupt the cell membrane. Disrupted cell suspension was centrifuged at 10,000 x g for 20 minutes and the supernatant was saved as the cytosolic fraction. Pelleted nuclei were resuspended in 1 PCV of buffer C (20 mM HEPES pH 7.9, 1.5 mM MgCl₂, 0.42 M NaCl, 0.2 mM EDTA, 25% (v/v) glycerol, 0.5 mM DTT, 1 µg/ml leupeptin, 2 µg/ml aprotinin, 1 µg/ml pepstatin A and 0.5 mM PMSF) and disrupted by drawing up and ejecting the mixture 10 times through a 26-gauge needle. Complete lysis of nuclei was achieved by incubation on ice and vortexing every 5 minutes for 15 seconds over a 30-minute period. The nuclear suspension was centrifuged at 20,000 x g for 5 min and the supernatant was saved as the nuclear extract.

Results and Discussion

Tissue expression of MBNL3 in the adult animal

Using RT-PCR, we examined the tissue expression profile of MBNL3 using total RNA isolated from 10 different adult mouse tissues. The MBNL3 transcript undergoes extensive alternative splicing in human cells [3,4]. Therefore, mouse-specific primers were designed against a homologous region of the human MBNL3 sequence that is conserved between all splice variants. In order to avoid amplification of contaminating genomic DNA, we designed the sense and antisense primers in the second and third exons of the mouse sequence, respectively. Primers designed to detect the ubiquitously expressed, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) transcript were used as a control for total input RNA.

We found that MBNL3 mRNA was enriched in the lung, spleen, and testis, and was absent or present in very low abundance in the brain, kidney, heart and skeletal muscle (Fig. 1). Intermediate expression levels were detected in the liver. This pattern resembles the findings of Kanadia et al, who reported that MBNL3 is expressed at very low levels in most adult mouse tissues [20]. In humans, MBNL3 was most prevalent in the placenta [4]. Intriguingly, these tissues along with the lung and spleen are highly vascularized. The low level of MBNL3 transcripts in differentiated muscle tissue is consistent with its role as an inhibitor of myogenesis. The lack of MBNL3 in other highly differentiated tissues suggests that the protein may play a more general antagonistic role in mammalian cell differentiation.

More recently, *MBNL* genes from the tiger pufferfish (*Takifugu rubripes*) were cloned and characterized [21]. In contrast to mammalian *MBNL3*, *tmbnl3* was highly expressed in all tissues, and was most abundant in the heart, testes, skin, and skeletal muscle. Unlike mammals, tiger pufferfish show indeterminate growth. Thus myogenic precursor cells are continually required to support myotube formation and the increase in myofiber diameter necessary for further growth. The constant presence of muscle progenitors could account for the high levels of *tmbnl3* expression in *T. rubripes* adult tissue. These data suggest that *MBNL3* is a potential marker of actively dividing precursor cells, consistent with the observation *MBNL3* is most prevalent in the liver, spleen and testes, sites of active proliferation in mammals.

MBNL3 expression levels differ between myogenic and nonmyogenic cells

MBNL3 was cloned by suppressive subtraction hybridization from actively proliferating ts13 myofibroblasts [18]. ts13 cells are a fibroblast-like cell line that can be induced to express muscle specific genes and adopt characteristics of myogenic cells [22]. *MBNL3* is highly homologous to muscleblind, a protein that is essential for muscle formation in flies. Therefore, we compared the expression level of *MBNL3* in ts13 myofibroblasts and C2C12 mouse myoblasts, a muscle precursor cell line that can be induced to differentiate in culture. As expected, *MBNL3* was detected in C2C12 cells by western blotting under growth conditions (Fig. 2A, lane 1). ts13 cells are derived from baby hamster kidney [23]. The antibody used for western blotting was generated against the mouse *MBNL3* protein. Fortunately, sufficient sequence similarity exists between the mouse and hamster proteins such that our anti-mouse *MBNL3* polyclonal antibody recognized the hamster polypeptide (Fig. 2A, lane 3). However, it is difficult to make any conclusions about the relative levels of *MBNL3* in C2C12 and ts13 cells due to potential differences in antibody affinity. What is evident is that *MBNL3* is expressed in both cell types.

Multiple polypeptides were detected by the *MBNL3* polyclonal antibody in ts13 hamster cell extracts (Fig. 2A, lane 3). The most abundant protein in ts13 cells was significantly smaller in size than the major protein found in C2C12 cells. The *MBNL3* transcript is subject to alternative splicing, with as many as 6 different isoforms identified for the human protein to date [3]. A search of the mouse database reveals a similar collection of mouse *MBNL3* splice variants (K.S. Lee, unpublished). Therefore alternative splicing of *MBNL3* is expected in hamster cells. However, we did not anticipate that the number of detectable *MBNL3* splice products would be greater in ts13 than in C2C12 cells (Fig. 2A, lanes 1 and 3). Whether the isoforms of *MBNL3* are functionally distinct has yet to be addressed.

Next, we examined the expression level of *MBNL3* in 10T1/2 mouse fibroblasts. In comparison to C2C12 cells, 10T1/2 fibroblasts expressed *MBNL3* at significantly lower levels (Fig. 2B). *MBNL3* expression in NIH3T3 fibroblasts also was extremely low and nearly undetectable by western blotting (data not shown). We conclude that the reduced levels of *MBNL3* are a general property of fibroblasts. Quantitative RT-PCR revealed that in C2C12 cells *MBNL3* mRNA levels were approximately 5- and 10-fold higher than in 10T1/2 and NIH3T3 cells, respectively (Fig. 2C). These results demonstrate that *MBNL3* expression differs between cell types and that *MBNL3* protein is not a ubiquitously expressed protein.

MBNL3 expression in mouse myoblasts and fibroblasts under differentiation conditions

We have observed that *MBNL3* expression decreased when C2C12 cells were induced to differentiate [18]. This observation prompted us to examine if *MBNL3* protein and mRNA levels change in ts13 and 10T1/2 cells in response to differentiation conditions. For these studies, cells were plated into growth media (DMEM +10% fetal bovine serum), and allowed to proliferate until they reached 70–80% confluency. Whole cell lysates were prepared from half the plates and the remaining cells were switched into differentiation media (DMEM + 2%

horse serum + ITS). After 0, 2, and 4 days of differentiation, whole cells extracts were prepared and subjected to SDS-polyacrylamide gel electrophoresis and anti-mouse MBNL3 western blotting. As expected, in C2C12 and ts13 cells, MBNL3 protein levels decreased under differentiation conditions (Fig. 2A and B). A reduction in 10T1/2 cells also was observed (Fig. 2B). Because fibroblasts do not undergo muscle differentiation, the decrease in MBNL3 expression most likely is due to the activation of signaling pathways resulting from the change in serum concentration (10% fetal bovine to 2% horse). Quantitative real-time PCR results (Fig. 2C) suggest that the mechanism of repression, in part, involves transcriptional and/or post-transcription regulation of MBNL3 mRNA levels.

MBNL1 expression in myoblasts and fibroblasts

MBNL1 is thought to function as a positive regulator of muscle differentiation while MBNL3 acts as an inhibitor. We examined the expression pattern of MBNL1 in C2C12 mouse myoblasts and two mouse fibroblast cell lines (NIH3T3 and 10T1/2) under growth and differentiation conditions. By western blotting, we detected comparable levels of MBNL1 in all three cell lines examined (Fig. 3A). In contrast to MBNL3, MBNL1 protein levels did not change when the cells were shifted to DM. These data are in conflict with results previously reported by Miller et al [17]. Under our experimental conditions, the regulation of MBNL1 and MBNL3 expression appears to involve distinct mechanisms.

Relative expression levels of MBNL1 and MBNL3 in myoblasts and fibroblasts

Different antibodies were used for the detection of MBNL1 and MBNL3, which does not allow for a direct comparison of their protein levels. Therefore, we turned to quantitative real-time RT-PCR to monitor the transcript levels of MBNL1 and MBNL3 in myoblasts and fibroblasts under growth and differentiation conditions. After correcting for differences in primer efficiency, all cell lines examined (C2C12, 10T1/2 and NIH3T3) expressed significantly higher levels of *MBNL1* mRNA than *MBNL3* mRNA (Fig. 3B). In NIH3T3 cells, the difference in message levels was greater than 100-fold.

To demonstrate that under our culture conditions C212 cells were undergoing muscle differentiation, we monitored the expression levels of two myogenic markers, myogenin and myosin heavy chain (MHC), which are induced during the early and late stages of muscle differentiation, respectively (Fig. 3C). High levels of myogenin were detected after 2 days in differentiation media while 4 days were required for MHC induction. The increase in muscle-specific protein expression was accompanied by a decrease in MBNL3 protein levels; however, MBNL1 protein levels remained unchanged (Fig. 3C). The contrast in expression patterns and protein levels in myogenic cells (C2C12) suggests that MBNL1 and MBNL3 perform unique functions during the muscle differentiation program.

MBNL3 is a nuclear protein in myoblasts and fibroblasts

Constitutive expression of MBNL3 in muscle precursor cells inhibits the induction of myogenic proteins during muscle differentiation. Determining the subcellular localization of MBNL3 will provide some insights into its potential mechanism of action. Using standard biochemical techniques, nuclear and cytosolic extracts were prepared from C2C12, ts13 and NIH3T3 cells maintained in growth media. The presence of MBNL3 in different subcellular fractions was determined by western blotting (Fig. 4). Nuclear TATA binding protein (TBP) and cytosolic α -tubulin were followed to assess the purity of the subcellular fractions. In all cell lines analyzed, MBNL3 was found predominantly in the nuclear fraction. A significant amount of MBNL3 was detected in the cytosol of ts13 cells. However, this signal can be attributed to the large amount of nuclear contamination, evident by the significant TBP signal in the ts13 cytosolic fraction (Fig. 4, lane 2). These findings have led us to propose that the primary site

of action for MBNL3 is in the nucleus of mammalian cells, where the protein can function as a negative regulator of muscle gene expression.

In summary, MBNL3 and MBNL1 respond differently to differentiation conditions in culture, suggesting that distinct signaling pathways regulate their expression in myoblasts and in fibroblasts. Additional studies are needed to elucidate the molecular mechanism that negatively regulates MBNL3 expression and the precise role of MBNL proteins during muscle differentiation.

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Fig 1. Expression of *MBNL3* mRNA in adult mouse tissues

(Upper panel) RT-PCR analysis (GibcoBRL One-Step RT-PCR kit) of the indicated mouse tissues were performed using 500 ng total RNA and primers designed against a 280 bp trans-exon region of the mouse *MBNL3* gene. (Lower panel) RT-PCR synthesis of the housekeeping gene, GAPDH, was performed with 1 ng total RNA to control for reverse transcriptase efficiency and RNA input.

Fig 2. Expression of MBNL3 is down-regulated under muscle differentiation conditions in myoblasts and in fibroblasts

(A) Whole cell lysates (100 μ g) derived from C2C12 myoblasts or ts13 myofibroblasts were subjected to 12% SDS-PAGE and MBNL3 was detected by western blotting. Cells were maintained in either growth (GM, lanes 1 and 3) or differentiation (DM, lanes 2 and 4) media for 48 hours before whole cell lysates were prepared. (B) C2C12 myoblasts and 10T1/2 fibroblasts maintained in growth media were switched to differentiation conditions. After 0, 2 or 4 days of differentiation, whole cells lysates were prepared and MBNL3 was detected by western blotting. (C) Transcript levels of MBNL3 were monitored by quantitative RT-PCR from the indicated cell lines cultured in growth (GM) or differentiation (DM) media for 48 hours. MBNL3 mRNA levels are expressed relative to the amount of message detected in C2C12 cells cultured in GM, which was assigned a value of 1.0.

Fig 3. MBNL1 expression pattern differs from MBNL3 during muscle differentiation

(A) Whole cell lysates were prepared from C2C12 myoblasts and two fibroblasts cell lines (10T1/2 and NIH3T3) after the cells were maintained for 0, 2, or 4 days in differentiation media (DM). The level of MBNL1 expression was determined by western blotting. α -tubulin levels were monitored to control for differences in protein loading. (B) Total RNA was isolated from C2C12, 10T1/2 and NIH3T3 cells that were cultured for 0 (G) or 2 (D) days under differentiation conditions. MBNL3 and MBNL1 transcript levels were determined by SYBR Green real-time PCR. The data is from one representative experiment carried out in triplicate. (C) Whole cell lysates were prepared from C2C12 cells maintained in differentiation media for 0, 2 and 4 days. The expression levels of MBNL3, MBNL1 and the differentiation markers myogenin and myosin heavy chain (MHC) were determined by western blotting. α -tubulin was used as a protein loading control.

Fig 4. MBNL3 protein fractionates to the nucleus of mammalian cells

Cytosolic and nuclear extracts were prepared from the indicated cell lines as described in Materials and methods. Proteins in each fraction were separated on SDS-polyacrylamide gel and the presence of MBNL3 was determined by western blotting using the anti-MBNL3 polyclonal antibody. The nuclear TATA binding protein (TBP) and cytosolic α -tubulin were monitored to assess the purity of the subcellular fractions.