The requirement of Mettl3-promoted MyoD mRNA maintenance in proliferative myoblasts for skeletal muscle differentiation

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1. Introduction

Stem cells are able to self-renew and to multiply rapidly while maintaining their pluripotency, resulting in histogenesis [1,2]. The differentiation process from stem cells to various tissues has previously been elucidated by studies that characterized the factors or conditions required for differentiation into each organ or tissue [3–9]; by contrast, it is unclear how the differentiation potential is maintained in proliferative cells. In the case of skeletal muscle, which accounts for approximately 40% and 30% of adult male and female body weights, respectively [10], it is reasonable to assume that the differentiation of stem cells and progenitors requires multiple rounds of cell division to attain a high muscular density. However, the means by which the myogenic potential is maintained in proliferative skeletal muscle stem cells and progenitors remains to be determined.

The identification of MyoD and the MyoD family of myogenic regulatory factors (MRFs) has revealed that the myogenic potential is determined by the expression of transcription factors that influence myogenic gene expression. Conversely, the loss of MyoD and/or MRFs in skeletal myoblasts, both in vitro in C2C12 cells [11,12] and in vivo, suppresses skeletal myogenesis...
During differentiation, together with Pbx homeodomain protein, MyoD binds to the regulatory regions of myogenic genes, including Myog, MyoD, MyoB, Timm and Ryr1 [15], which triggers chromatin remodelling by recruiting the SWI/SNF chromatin remodelling factor BrG1 for myogenic gene transcription [16,17]. Before differentiation, MyoD labels myogenic gene loci by incorporating the variant histone H3.3 with chromodomain helicase DNA-binding domain 2 (Chd2), without activating transcription [8]. Thus, during proliferative phases, MyoD is critical for myoblast differentiation and the inheritance of differentiation potency.

Transcription factor expression is destabilized by RNA modifications that influence the differentiation potential of stem cells [18,19]. In embryonic stem (ES) cells, methyltransferase like 3 (Mettl3) [20] induces an N^2-methyadenosine (m^2A) modification in Nanog RNA, which is required for pluripotency and differentiation [19]. m^2A modifications have been reported to affect RNA function by various mechanisms, including splicing, stabilization/destabilization [18,21], nuclear export [22] and translation efficiency [23,24]. RNA modifications or stabilization may also be crucial for skeletal muscle differentiation because MyoD mRNA has a short half-life of approximately 90 min [25,26]. The RNA-binding protein HuR has been reported to stabilize MyoD mRNA and to be necessary for terminal skeletal muscle differentiation [25,27]. It was also shown that MyoD mRNA levels are quite low in G0-arrested cells but increase upon re-entry into the cell cycle [28], implying that MyoD mRNA could be stabilized during the cell cycle. However, the underlying mechanisms, as well as the factors required for the maintenance of MyoD expression during proliferation, remain to be clarified.

Here, we explored the maintenance of MyoD mRNA levels in proliferative myoblasts. We found that cell cycle arrest reduced MyoD mRNA expression, thus suppressing myogenic differentiation, and that Mett3 stabilized MyoD mRNA by promoting mRNA processing in skeletal myoblasts. Our results suggest that m^2A modification by Mett3 stabilizes MyoD mRNA levels for skeletal muscle differentiation.

2. Results

2.1. Cell cycle arrest in S and G2 phases reduces MyoD mRNA levels and inhibits myoblast differentiation

During proliferative phases, MyoD binding to target genes such as myogenic genes is required for skeletal muscle differentiation [8,13,29], suggesting that the maintenance of MyoD expression during cell cycle progression could be critical for differentiation. Because MyoD mRNA levels were reported to be low following cell cycle arrest at G0 [28], we hypothesized that cell cycle arrest may cause MyoD mRNA instability. C2C12 cells, a mouse myoblast cell line with both self-renewal and differentiation potential, were arrested either in the S phase by thymidine or in the G2 phase by the Cdk1 inhibitor RO-3306, and MyoD mRNA levels were analysed by quantitative reverse transcription PCR (qRT-PCR). Cell cycle arrest was confirmed by measuring the population in each cell cycle phase after exposure to thymidine or RO-3306 (electronic supplementary material, figure S1a). We observed cell cycle re-entry and the proliferation of cells after removal of the inhibitors (electronic supplementary material, figure S1b), confirming that the drug treatments did not induce cell death under our experimental conditions. qRT-PCR analysis showed that MyoD mRNA levels were significantly reduced after cell cycle arrest in both S and G2 phases in the growth state (p = 0.04 and 0.007, respectively; figure 1a(i)). On the other hand, mRNA levels of other skeletal muscle-specific transcription factors (Pax7 and Srf) were not substantially affected by cell cycle arrest in the proliferative state (electronic supplementary material, figure S2a).

Next, we examined the skeletal muscle differentiation of arrested C2C12 cells. Cells were cultured with 2% horse serum (HS) media for 48 h to induce differentiation. We found that myotube formation was diminished in thymidine- and RO-3306-treated cells but not in mock-treated cells (figure 1b). qRT-PCR analysis revealed that mRNA levels of MyoD as well as those of the skeletal muscle-specific genes Myog and Acta1 were decreased by cell cycle arrest in the differentiated state.

Figure 1. MyoD mRNA expression is reduced upon cell cycle arrest. (a) mRNA levels of Myod1, Mettl3 and Elavl1 in C2C12 cells treated with thymidine or RO-3306 for 48 h. (i) Cells treated with thymidine were compared with non-treated cells. (ii) Cells treated with RO-3306 were compared with DMSO-treated cells. (b) Morphology of C2C12 cells treated with DMSO (labelled as Mock), thymidine and RO-3306. RO-3306 was used as a Cdk1 inhibitor. Cells were immunostained for anti-MyHC and Hoechst at 72 h after the induction of differentiation. MyHC, myosin heavy chain.
Taken together, these results suggest that during proliferative phases, cell cycle arrest leads to a reduction of MyoD mRNA expression that is required for skeletal muscle differentiation.

2.2. Cell cycle arrest affects mRNA levels of HuR and Mettl3

To examine the mechanism by which MyoD mRNA expression is decreased upon cell cycle arrest, we focused on two pathways potentially involved in RNA metabolism. One was the HuR (also known as Elavl1)-mediated stabilization of RNA, which involves HuR binding to AU-rich elements of MyoD mRNA in the early stages of skeletal myoblast differentiation [25,27,30]. The other was the m^6^A modification of RNA introduced by Mettl3 [20], which is also important for RNA stabilization. qRT-PCR analysis showed that Elavl1 and Mettl3 mRNA levels were significantly reduced by cell cycle arrest using thymidine (both p < 0.05; figure 1a(i)), but the effect was relatively limited following treatment with RO-3306 (p = 0.087 and 0.143, respectively; figure 1a(ii)). These results suggest that the decreased expression of HuR and/or Mettl3 may account for the decline in MyoD mRNA expression upon cell cycle arrest.

2.3. Knockdown of Mettl3, but not of HuR, downregulates MyoD mRNA levels in skeletal myoblasts

Next, to determine if HuR and/or Mettl3 regulate MyoD mRNA expression, we carried out small interfering (si)RNA-mediated knockdown of Mettl3 and HuR. qRT-PCR showed that MyoD mRNA levels were significantly reduced in Mettl3 knockdown cells (p = 0.03; figure 2a(i)),

Figure 2. Knockdown of Mettl3, but not HuR, downregulates MyoD mRNA levels and suppresses skeletal muscle differentiation. (a) qRT-PCR analysis of Myod1 in (i) Mettl3 and (ii) HuR knockdown cells. (b) qRT-PCR analysis to monitor (i) Mettl3 and (ii) HuR knockdown efficiency. (c) Western blot analysis to monitor (i) Mettl3 and (ii) HuR knockdown efficiency. Hsp90 was used as a loading control. (d) Morphology of C2C12 cells with transfected Mettl3 siRNA. Cells were immunostained for anti-MyHC and Hoechst at 72 h after the induction of differentiation.
but not in HuR knockdown cells (figure 2a(ii)). Both siRNAs suppressed the expression of target genes at the mRNA (figure 2b) as well as protein level (figure 2c), validating the knockdown efficiency. These data suggest that Mettl3, but not HuR, is implicated in maintaining MyoD mRNA levels. Mettl3-targeted siRNA treatment also suppressed myotube formation (figure 2d).

2.4. Knockdown of Mettl3 markedly downregulates processed, but not unprocessed, MyoD mRNA levels

Because m^6A modification by Mettl3 has been proposed to regulate RNA metabolism such as splicing [31–35], we examined the processing of MyoD pre-mRNA for the generation of mature MyoD mRNA. To investigate whether Mettl3 affects MyoD RNA splicing, two primer pairs were designed to anneal between two of the three MyoD exons and another two pairs to include both an intron and an exon (figure 3a).

qRT-PCR analysis showed that in Mettl3 knockdown cells, fewer PCR products containing only exons were obtained (figure 3b(ii)), whereas there was no effect on the amount of PCR products containing an intron (figure 3b(i)). The length of PCR products amplified with exonic primer pairs was the correct splice size, and non-spliced RNA was not amplified (electronic supplementary material, figure S3a). Taken together, this indicates that knockdown of Mettl3 significantly decreased the amount of processed MyoD mRNA without affecting that of unprocessed MyoD RNA.

To investigate whether the m^6A reading process is associated with the maintenance of MyoD mRNA, we focused on the representative m^6A readers YTHDF2, YTHDC1, HNRNPA2B1 and HNRNPC [18,24,31–36], which were previously shown to be involved in RNA stability and processing [34,35]. We performed siRNA-mediated knockdown of these four factors and found that MyoD mRNA expression and myotube formation were only suppressed by Ythdf2 knockdown (electronic supplementary material, figure S3b,c). Knockdown of Ythdf2, however, led to a decrease of both processed and unprocessed MyoD RNA (electronic supplementary material, figure S3d), unlike the case of Mettl3 knockdown.
m6A modifications of MyoD mRNA are primarily enriched in the 5' untranslated region

To further investigate the m6A modification of Myod1 mRNA, m6A-sequencing (m6A-seq) was conducted by pulling down transcripts with an antibody that specifically recognizes m6A modifications [31]. Published studies have reported that m6A modifications are distributed in 3' UTRs, stop codons and internal long exons, but rarely in 5' UTRs [19,31,37,38] (figure 4a(ii)), while we observed enriched m6A signals in C2C12 cells both near the start codon and stop codon (figure 4a(i)). Integrative Genomics Viewer plots showed that while m6A modifications on the majority of RNAs such as Gapdh and Srf were enriched around 3' UTRs, stop codons and long exons (figure 4b; electronic supplementary material, figure S4), m6A modifications of Myod1 were notably enriched around the 5' UTR (figure 4b(i), green arrow).

We also found that C2C12 cells and ES cells had many analogous loci for m6A signal enrichment (figure 4b(iii); electronic supplementary material, figure S4, upper panel). However, several skeletal muscle-specific genes such as Myod1 and Myog were enriched in m6A signals only in C2C12 cells, whereas m6A signal enrichment in pluripotent genes such as Nanog was observed only in ES cells (figure 4b(i); electronic supplementary material, figure S4, middle and lower panels), reflecting cell type-specific regulations.

m6A modifications of Myod1 mRNA during cell proliferation

To investigate the function of m6A modification on MyoD mRNA, especially around the 5' UTR, we constructed vectors harbouring Myod1 with various mutations and deletions in the 5' UTR. 'RRAC' has been shown to be a common sequence of m6A sites, with 'GGAC' the most frequent of these [18,24,31,37,38]. m6A-seq revealed the presence of some 'GGAC' motifs within m6A modifications on Myod1 5' UTR. Therefore, deleted or mutated versions of three 'GGAC' motifs in the Myod1 5' UTR were generated, as well as an entire 5' UTR deletion mutant, and an entire 3' UTR deletion mutant (figure 5a). These mutants were transiently transfected into NIH3T3 cells, and qRT-PCR detected total (both processed and unprocessed) and processed MyoD mRNA using primer pairs within exon 1 of Myod1 and between exon 1 and exon 2, respectively.

The ectopic introduction of each vector could be monitored by total MyoD RNA levels, all of which led to a similar pattern
The function of m6A is largely dependent on modifications reported that m6A is more commonly seen in 3' UTRs and that m6A promotes cap-independent translation by recruiting splicing factors \([32,33]\). Our m6A-seq data showed that MyoD mRNA levels were analysed at days 0, 3, 6 and 12 using a primer set within the stop codon region (42%), the coding region (36%) and the 3' UTR (80%). Between additional doses, reactivated cells may have affected mRNA levels, which may explain the more prominent effects of thymidine compared with RO-3306. To directly examine the involvement of HuR and Mettl3 in the regulation of MyoD mRNA expression, we conducted siRNA-mediated knockdown experiments and found that knockdown of Mettl3, but not of HuR, significantly affected MyoD mRNA expression. Previous studies reported that target RNAs for YTHDF2 are destabilized by m^6^A modification and stabilized upon Mettl3 knockdown [18,19]. Our results, however, showed the opposite effects, and thus suggest that MyoD mRNAs are unlikely to be a direct target of YTHDF2. This raises the possibility that YTHDF2 functions to indirectly stabilize MyoD mRNA through its many (greater than 3000) targets [18]. Future work should attempt to uncover the mechanisms involving these factors.

Although HuR and Mettl3 mRNA expression was reduced by thymidine-mediated cell cycle arrest, this decrease was non-significant following treatment with RO-3306. Cell cycle arrest was planned for the standard duration of 48 h. While a single treatment of thymidine was adequate to achieve cell cycle arrest because its effects increase gradually for at least 48 h, RO-3306 acts rapidly within 8 h, but its effects are diminished by 12 h such that additional treatments were required at intervals of 8–12 h. Between additional doses, reactivated cells may have affected mRNA levels, which may explain the more prominent effects of thymidine compared with RO-3306. To directly examine the involvement of HuR and Mettl3 in the regulation of MyoD mRNA expression, we conducted siRNA-mediated knockdown experiments and found that knockdown of Mettl3, but not of HuR, significantly affected MyoD mRNA expression. Previous studies reported that HuR stabilizes MyoD mRNA by binding to its AU-rich elements during the early stages of differentiation [25]. The discrepancy between these results and our own remains to be resolved. Because our investigation was conducted during the growth state, HuR may have had little effect on MyoD mRNA expression before the onset of differentiation. Interestingly, it was previously reported that HuR is significantly associated with m^6^A bait [31], but the exact relationship remains to be elucidated.

In summary, MyoD mRNA levels appear to be maintained by Mettl3-mediated m^6^A modifications, suggesting that Mettl3 is a critical regulator for skeletal muscle differentiation. These findings help understand the mechanisms underlying the maintenance of myogenic potential in proliferative skeletal muscle progenitors. Moreover, the methods developed in this study will be useful for future research in this area.
study may be beneficial in evaluating different cell types from various species.

4. Material and methods

4.1. Cell culture and drug treatment

The mouse myoblast cell line C2C12 was maintained in a growth state in Dulbecco's modified Eagle's medium (DMEM) (Gibco by Thermo Fisher Scientific) containing 1 g l\(^{-1}\) glucose, 1% l-glutamine and 1% penicillin/streptomycin supplemented with 20% fetal bovine serum (FBS). NIH3T3 cells were maintained in DMEM containing 4.5 g l\(^{-1}\) glucose, 1% l-glutamine and 1% penicillin/streptomycin supplemented with 20% FBS. Cells were grown in a 5% CO\(_2\) cell culture incubator at 37°C. Differentiation stimuli were induced by exchanging media with 2% HS instead of 20% FBS.

C2C12 myoblasts were arrested in S phase by thymidine (Sigma-Aldrich, T1895) and in G2 phase by Cdk1 inhibitor (RO-3306, Sigma-Aldrich, SML0569) for 48 h. A single treatment of thymidine was applied, while additional RO-3306 was added at intervals of 8–12 h.

4.2. Immunocytochemistry

Cells were cultured on µ-Plate 24 Well (ibidi), washed twice with phosphate-buffered saline (PBS), fixed with 1% paraformaldehyde in PBS, permeabilized with 0.5% Triton X-100 in PBS and washed twice with PBS. A 15 min incubation with Blocking One (Nacalai Tesque Inc.) was followed by a 2 h incubation with mouse anti-myosin heavy chain (MF20, eBioscience, 1 : 200) diluted with 10% Blocking One in PBS at room temperature. The µ-Plate 24 Well was then washed three times with PBS and incubated for 30 min at room temperature with CF568-labelled goat anti-mouse antibody (1 : 100; Biotium Inc.) and Bisbenzimide H33342 Fluorochrome (RO-3306, Sigma-Aldrich, SML0569) for 48 h. A single treatment of thymidine was applied, while additional RO-3306 was added at intervals of 8–12 h.

4.3. Fluorescence activated cell sorting

C2C12 cells in a growth state were isolated by trypsin treatment and centrifuged for 5 min at 190 g. The cell pellet was resuspended in 1 ml of PBS supplemented with Hoechst 33342 stain (Nacalai Tesque Inc.) and ethanol precipitation. Total RNA was extracted from cells using Sepasol-RNA I Super G (Nacalai Tesque Inc.) and ethanol precipitation.

4.4. Quantitative RT-PCR

Total RNA was extracted from cells using Sepasol-RNA I Super G (Nacalai Tesque Inc.) and ethanol precipitation. Total RNA (1 µg) was used for reverse transcription with the PrimeScript RT reagent kit (Takara Bio Inc., RR047A). qRT-PCR was performed using Thunderbird SYBR qPCR Mix (Toyobo Co., Ltd.) with the PikoReal 96 Real-Time PCR System (Thermo Fisher Scientific) as previously described [40]. Primers are listed in the electronic supplementary material, table S1. qRT-PCR data were normalized to Gapdh or Eef1a1 expression levels and presented as the mean ± s.d. of three independent experiments. For qRT-PCR of processed MyoD mRNA in NIH3T3 cells with transfected MyoD mutants, differences in transfection efficiencies were normalized by using total MyoD RNA in each sample as an internal control. Total MyoD RNA levels were quantified by performing qRT-PCR using primer pairs within exon 1 of MyoD, while processed MyoD mRNA was quantified using primer pairs between exon 1 and exon 2.

4.5. Western blotting

Cells were harvested and disrupted in a Sample Buffer Solution with 2-ME (Nacalai Tesque Inc.). Samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis using a 5–20% Extra PAGE One Precast Gel (Nacalai Tesque Inc.) and electrotransferred to a polyvinylidene fluoride membrane with the Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, Hercules, CA; 2.5 A, 25 V, 7 min). The membrane was blocked for 1 h in 5% (w/v) skimmed milk in Tris-buffered saline containing 0.05% (v/v) Tween 20, then incubated with primary antibodies in Hikari Solution A (Nacalai Tesque Inc.), followed by incubation with secondary antibodies and detection using Chemi-Lumi One Ultra (Nacalai Tesque Inc.).

The following primary antibodies were used for western blotting: rat anti-MyoD (SF11, Millipore 1 : 500), anti-Mettl3 (15073-1-AP, Proteintech, 1 : 1000), anti-Hsp90 (H-114, Santa Cruz Biotechnology, 1 : 1000) and mouse anti-HuR (3A2, Santa Cruz Biotechnology, 1 : 200). Secondary antibodies were horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG antibodies (GE Healthcare, 1 : 5000).

4.6. siRNA-mediated knockdown of Mettl3, Elavl1 and m\(^6\)A readers

Transient knockdown of target genes by siRNA was performed with Lipofectamine RNAi MAX (Invitrogen) and opti-MEM (Gibco by Thermo Fisher Scientific). The following siRNAs were used: siMettl3 (Dharmacon, ON-TARGETplus SMARTpool #L-049446), siElavl1 (Dharmacon, ON-TARGETplus SMARTpool #L-053812), stYthd2 (Dharmacon, ON-TARGETplus SMARTpool #L-058271), stYthd1 (Dharmacon, ON-TARGETplus SMARTpool #L-167076), siHnrnpa2b1 (Dharmacon, ON-TARGETplus SMARTpool #L-040194) and siHnrnpc (Dharmacon, ON-TARGETplus SMARTpool #L-044147).

4.7. Transfection

Seven MyoD mutants were generated as DNA inserts. Each insert DNA was amplified by PCR using KOD FX neo DNA polymerase (Toyobo Co., Ltd.) and ligated with the pCAGGS vector. A list of primers for cloning is shown in the electronic supplementary material, table S1. Plasmid transfection was performed using Lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions. The sequences of all mutants were confirmed by Sanger sequencing.

4.8. mRNA-seq

Total RNA was extracted from cells using Sepasol-RNA I Super G (Nacalai Tesque Inc.) and ethanol precipitation.
Purified RNA samples underwent library construction using the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs, NEB #7420S). Library preparation and sequence analysis followed a previously described protocol [41].

Sequenced reads were mapped onto the mouse genome (mm9) using TopHat (version 2.0.8). Gene expression levels (fragments per kilobase of exon per million mapped sequence reads) were estimated using the Cuffdiff program in CUFFLINKS (version 2.0.1) using mapped reads and the software’s default parameters [42].

4.9. m6A immunoprecipitation

m6A immunoprecipitation was performed according to a previously described protocol [31,43]. Total RNA was extracted from cells with Sepasol-RNA I Super G (Nacalai Tesque) and ethanol precipitation, and 348 µg was used for independent experiments. RNA was fragmented by mixing with 10 × fragmentation buffer (1 M ZnCl2, 1 M Tris–HCl pH 7.0 and RNase-free water) at 94°C for 5 min using a preheated thermal cycler. The fragmentation mix was ethanol precipitated by adding 1/10 volumes of 3 M sodium acetate, pH 5.2, glycogen (100 µg ml-1 final), and 2.5 volumes of 100% ethanol, mixing well, then incubating at –80°C overnight. Fragmented RNA was mixed with an anti-m6A antibody (Synaptic Systems, 202003, 12.5 µg) and 5 × immunoprecipitation buffer (0.05 M Tris–HCl, pH 7.4, 0.75 M NaCl, 0.5% Igepal CA-630, and RNase-free water). Ribonucleoside-vanadyl complexes (Sigma-Aldrich, R3380, 2 mM final) and RNasin Plus RNase inhibitor (Promega, N2611, 200–400 U, final) were mixed with immunoprecipitation reagents to prevent RNA degradation. Immunoprecipitation mixtures were incubated for 2 h at 4°C on a rotating wheel to allow the formation of antibody-RNA complexes. These complexes were mixed with Dynabeads Protein A (Novex by Life Technologies) and incubated for 2 h at 4°C on a rotating wheel. Immunoprecipitated RNA was eluted by competition with N6-methyladenosine, 5’-monophosphate sodium salt (m6A, Sigma-Aldrich, M2780, 6.7 mM final).

4.10. m6A-seq data analysis

Purified RNA fragments from m6A RNA immunoprecipitation underwent library construction using the NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs, NEB #7420S). The m6A-seq library was sequenced on an Illumina HiSeq 1500 sequencing system. Sequence reads were aligned to the reference mouse genome (GRCm38) using HISAT2 software (version 2.0.4) [44]. The software DEEPTOOLS2 (version 2.3.5) [45] was used to create the coverage tracks (BigWig file) of m6A-modified/input RNA on the mouse genome with the options: bamCoverage -binSize 1 -normalizeTo1x226726534 (the effective mouse genome size for 50 bp reads), and the m6A signal profiles on all coding sequences (taken from UCSC refSeq genes) with the options: computeMatrix -m 2000 -b 2000 -a unscaledSparse 500 -unscaledSparse 500 -skipZeros -missingDataAsZero.

4.11. Statistical analysis

Statistical significance in qRT-PCR data was evaluated using the two-sided Welch’s t-test.

Data accessibility. mRNA-seq and m6A-seq data were deposited into the DNA Data Bank of Japan database (accession no.: DRA005057). The other datasets supporting this article have been uploaded as part of the electronic supplementary material.

Authors’ contributions. K.K. and A.H. performed the experiments. J.N. and K.M. performed statistical analysis. K.K., T.K., H.S., E.O., Y.M. and Y.O. designed the experiments and data analysis. K.K., T.K. and Y.O. wrote the manuscript.

Competing interests. We have no competing interests.

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