A novel mechanism of myostatin regulation by its alternative splicing variant during myogenesis in avian species

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MYOSTATIN (MSTN), also known as growth and differentiation factor-8, is a member of the transforming growth factor-β superfamily and is well-known as a negative regulator of muscle growth and development. The myostatin gene (MSTN) is highly conserved in vertebrates, containing three exons and two introns. Myostatin is mainly synthesized in skeletal muscle; myogenesis; myostatin; alternative splicing; bird

Myostatin starts to be expressed in early embryonic stages, especially in the somites, which give rise to skeletal muscle as development progresses. For example, the expression of myostatin can be detected in the dermomyotome compartment of somites in day 10.5 mouse embryos (15). In cattle, the expression of myostatin can be detected at a low level during day 15 to 29 embryos and increases after day 31 (13). In pigs, myostatin starts to be detected in 21-day-old fetuses and significantly increases from day 49 to day 105 (10). In chicken, myostatin mRNA is detected as early as after oviposition and in the ventral myotomal region of somites during early embryogenesis (2, 22). Recently, alternative splicing isoforms of the myostatin gene have been reported in ducks and sheep (8, 9). However, conservation of myostatin splicing isoforms among avian species and their roles in muscle growth and development have not been investigated.

In the current study, we identified five alternatively spliced myostatin isoforms in various tissues and embryonic skeletal muscle cell cultures of avian species. In addition, full-length quail myostatin and a major alternative splicing form in muscle is not active under the latent myostatin complex, but this complex has been shown to circulate in the blood (30). Next, the cleavage of propeptides between Arg-75 and Asp-76 by the bone morphogenetic protein-1/tolloid family of metalloproteinases leads to disassociating the latent myostatin complex and releasing the active mature myostatin dimer (26, 30).

Because myostatin is a negative regulator of muscle growth and development, muscle hypertrophy can be achieved by inhibiting myostatin expression, activity, and signal cascade. In fact, the disruption of the myostatin gene in mice and overexpression of myostatin antagonist or dominant-negative myostatin receptors in transgenic mice resulted in a significant increase in muscle mass (14, 16, 25, 29). Overexpression of myostatin propeptide has a similar stimulatory effect on muscle growth in transgenic mice as shown in myostatin-knockout mice (28). In addition, mutations of myostatin have been identified as the cause for double muscling in many species including cattle, humans, and dogs due to both hyperplasia and hypertrophy (6, 7, 13, 16, 20, 21). Furthermore, reduced expression of myostatin by microRNAs also caused muscle hypertrophy in sheep (3). These studies clearly demonstrated the negative roles of myostatin in muscle growth and also perturbation of MSTN could enhance muscle mass, suggesting MSTN as a potential therapeutic target for diseases associated with muscle weakness and atrophy.

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In the current study, we identified five alternatively spliced myostatin isoforms in various tissues and embryonic skeletal muscle cell cultures of avian species. In addition, full-length quail myostatin and a major alternative splicing form in muscle

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tissue were overexpressed to investigate the biological role of alternative splicing variants of myostatin in muscle development and the mechanism of their interplay in regulating myogenesis.

**Glossary**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>aa</td>
<td>Amino acids</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-Diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DD</td>
<td>Days of differentiation</td>
</tr>
<tr>
<td>ED</td>
<td>Embryonic days</td>
</tr>
<tr>
<td>EV</td>
<td>Empty vector</td>
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<td>IP</td>
<td>Immunoprecipitation</td>
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<tr>
<td>MSTN</td>
<td>Myostatin</td>
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<tr>
<td>MyHC</td>
<td>Myosin heavy chain</td>
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<tr>
<td>PD</td>
<td>Post-hatch days</td>
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<tr>
<td>PM</td>
<td>Pectoralis major muscle</td>
</tr>
<tr>
<td>QM7</td>
<td>Quail muscle clone 7</td>
</tr>
<tr>
<td>RPS13</td>
<td>Ribosomal protein S13</td>
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<tr>
<td>TM</td>
<td>Thigh muscle</td>
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</table>

**MATERIALS AND METHODS**

**Experimental birds.** Animal care procedures were approved by the Institutional Animal Care and Use Committee of The Ohio State University. To investigate the tissue distribution of *MSTN*, various tissue samples, fat (F), pectoralis major muscle (PM), thigh muscle (TM), heart (H), liver (Li), lung (Lu), and kidney (K), were collected from three individuals for chicken (PD 21), turkey (PD 14), and quail (PD 21).

Total RNA extraction, cDNA synthesis, and PCR. Total RNA was isolated from various tissues of chicken, quail, and turkey. Tissue samples were homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA) by using a Tissuemiser homogenizer (Fischer Scientific, Pittsburgh, PA). Total RNA was isolated according to the manufacturer's protocol. After checking the quality and quantity, one microgram of total RNA was used for cDNA synthesis by reverse transcription using Moloney murine leukemia virus reverse transcriptase (Invitrogen). To analyze alternative splicing forms and expression patterns of *MSTN* in various tissues, two primers, forward primer on exon 1: 5'-AGC ACC TAA CAT TAG CAG GGA CGT-3' and reverse primer on exon 3: 5'-CAG ACC TGG GGT GCC ACC CTG-3', were designed to cover full-length *MSTN*. The amplification conditions for *MSTN* were 94°C for 1 min, 33 cycles of 94°C for 30 s, 58°C for 45 s, and 72°C for 40 s, and final extension time of 5 min at 72°C. PCR products were separated on a 2% agarose gel, purified, and then sequenced.

**Vector construction.** Two quail *MSTN* clones, *MSTN*-A and -B, were amplified by PCR from quail muscle cDNA. *MSTN*-A-Myc, containing the entire coding sequences for quail *MSTN* (GenBank no. AF407340.1), was amplified with a primer set of qMSTN/F as a forward primer and qMSTN-A-Myc/R as a reverse primer. *MSTN*-B-HA or -Myc containing the first exon and the first 14 bp of the second exon was amplified with qMSTN/F as a forward primer and qMSTN-B-HA/R or qMSTN-B-Myc/R as a reverse primer, respectively. The *MSTN*-pro-Myc, the prodomain of *MSTN*, was amplified with qMSTN/F as a forward primer and qMSTN-pro-Myc/R as a reverse primer. To construct the *MSTN*-de1-Myc, which has a deletion of exon 1 from full-length *MSTN*, two pieces excluding exon 1 were amplified with two primer sets, qMSTN/F and qMSTN-de1-PacI/R for the first piece and qMSTN-de1-PacI/F and qMSTN-A-Myc/R for the second piece, and these were connected together after PacI enzyme digestion and ligation into the pcDNA3.1 vector (Invitrogen). The *MSTN*-de2-Myc, which has a deletion of exon 2 from full-length *MSTN*, was constructed by ligation of two pieces of PCR product amplified with two primer sets, qMSTN/F and qMSTN-de2-AgeI/R for the first piece and qMSTN-de2-AgeI/F and qMSTN-A-Myc/R for the second piece, into the pcDNA3.1 vector after Agel enzyme digestion. PCR products were directly inserted into the T-overhang EcoRV site of pcDNA3.1 vector or after additional manipulations, such as enzyme digestion. The primers and their sequences used for vector construction are listed in Table 1.

**Primary cell culture.** Primary muscle cells were obtained from the PM muscles of a quail, chicken, and turkey (ED 11, 13, and 15, respectively) and were cultured as previously reported (27). Briefly, muscle tissues were minced in PBS and then digested thoroughly in 0.05% trypsin-EDTA at 37°C for 20 min. After being filtered through a 100 μm cell strainer (BD Biosciences, San Jose, CA), the cells were collected by centrifuging at 200 g for 5 min. Cells were then seeded in 35 mm tissue culture plates (TPP, St. Louis, MO) at an initial cell density of ~3.6 × 10⁴ cells per dish in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, Grand Island, NY), supplemented with 10% fetal bovine serum (FBS, Gibco), penicillin (100 U/ml) and streptomycin sulfate (100 μg/ml; Gibco). Differentiation was induced with differentiation medium [DMEM with 2% horse serum (Gibco)] when cells reached 80% confluence. Cells were collected at 0, 12, 24, 36, and 48 h for total RNA isolation, RT-PCR, and cell morphological images.

**QM7 cell line culture and stable cell line generation.** QM7 myoblasts were seeded at a density of 7 × 10⁴ cells/cm² in medium 199, supplemented with 10% tryptose phosphate broth (Sigma-Aldrich, St. Louis, MO), 10% FBS, and penicillin (100 U/ml) and streptomycin sulfate (100 μg/ml). Differentiation was induced when cells reached 90% confluence by changing the medium to 0.5% FBS. Three stable QM7 cell lines were obtained by simultaneous stable transfection of pcDNA3.1 containing no insert (empty vector, EV).

Table 1. Primer sequences for cloning quail *MSTN* full-length and fragments

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’-3’)</th>
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<tr>
<td>qMSTN/F</td>
<td>CTGGTTAAAGCTGCAAAATAATCGTAGCTG</td>
</tr>
<tr>
<td>qMSTN-A-Myc/R</td>
<td>CTCAACAGGCTTTCCTCCATGAGTCAGTTTTCGAGCCACCCAGAGCTCTAC</td>
</tr>
<tr>
<td>qMSTN-B-HA/R</td>
<td>CTGAAACGGCTAGCTCTCGGAAGCTGTATGAAATGATGGCTGATGTCAG</td>
</tr>
<tr>
<td>qMSTN-Myc/R</td>
<td>CTCAACAGGCTTTCCTCCATGAGTCAGTTTTCGAGCCACCCAGAGCTCTAC</td>
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<td>qMSTN-pro-Myc/R</td>
<td>CTCAACAGGCTTTCCTCCATGAGTCAGTTTTCGAGCCACCCAGAGCTCTAC</td>
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<td>qMSTN-de1-PacI/R</td>
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<td>qMSTN-de1-PacI/F</td>
<td>GTTAAATAAGACTGGAGCTGCAGGTCT</td>
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<td>qMSTN-de2-AgeI/R</td>
<td>CCGACCGTGCTCGAAGCTGATGAAATGAT</td>
</tr>
<tr>
<td>qMSTN-de2-AgeI/F</td>
<td>CCGACCGTGCTCGAAGCTGATGAAATGAT</td>
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*MSTN*, myostatin.
were cultured and differentiated. At 0, 2, and 4 days after differentiation for 2 days. Then, cells were lysed with RIPA buffer, and cell lysates were collected by centrifugation at 15,000 g. The supernatants were precipitated using trichloroacetic acid (TCA; 10% TCA; Santa Cruz Biotechnology, Dallas, TX) at 4°C for 2 h. Then, the pellets were washed five times with RIPA buffer, and proteins were eluted by adding 2× Laemmli sample buffer and incubating at 98°C for 5 min. The eluted proteins were used for immunoblotting to confirm protein binding.

Immunoblotting. QM7 cells were collected using ice-cold 1× lysis buffer (62.5 mM Tris, 5% SDS) or RIPA buffer. The coimmunoprecipitated proteins were used directly after elution. Cell medium proteins were precipitated using trichloroacetic acid (TCA; final concentration: 30%). Briefly, culture medium was centrifuged at 1,200 g to pellet cell debris. After adding the proper amount of TCA, medium was centrifuged at 12,000 g for 15 min. The pellets were washed three times with ice-cold acetone and then resolved in SDS loading buffer.

Equal amount of proteins were separated by 10 or 12% SDS-PAGE and wet-transferred to polyvinylidene fluoride membranes (Bio-Rad, Hercules, CA). After blocking with 5% nonfat milk dissolved in Tris-buffered saline-Tween (TBST; 20 mM Tris, 150 mM NaCl, pH 7.4, and 0.05% Tween 20) for 1 h, the membranes were incubated with the indicated antibodies for 1 h at room temperature. Rabbit anti-Myc-tag (1:5,000; Cell Signaling), and mouse anti-HA-tag (1:2000; Cell Signaling) antibodies were used as primary antibodies. Horseradish peroxidase-conjugated anti-rabbit IgG (1:5,000; Cell Signaling) and anti-mouse IgG (1:5,000; Cell Signaling) were used as secondary antibodies. Signals were detected with ECL Plus (GE Biosciences). The membranes were exposed to Bio-Max X-ray film (GE Biosciences) for visualization of target proteins.

Immunocytochemistry. QM7 cells (EV, MSTN-A, and MSTN-B) were cultured and differentiated. At 0, 2, and 4 days after differentiation, the cells were fixed with 10% formalin for 20 min at room temperature. After permeabilizing with 0.25% Triton-X100 in PBS and blocking with blocking buffer (1% BSA and 0.05% Tween-20 in PBS), cells were incubated with mouse anti-myosin heavy chain antibody (1:100; DSHB, Iowa City, Iowa, IA) in blocking buffer. Donkey anti-mouse antibody conjugated with FITC (1:200; The Jackson Laboratory) in blocking buffer was used as the secondary antibody. 4’,6-diamidino-2-phenylindole (DAPI, Invitrogen) was added to stain cell nuclei. Images were taken using an AXIO-Vert. A1 optical microscope (Carl Zeiss Microscopy, Thornwood, NY) and AxioCam MRc5 camera (Carl Zeiss Microscopy), and measurements of the cell length, width, density, fusion index (number of nuclei in the myotubes/total number of nuclei), and nuclei number per myotube were taken.

Statistical analysis. All of the experiments were independently repeated at least three times. Statistical analyses were performed using the SAS software system (SAS Institute, Cary, NC). Differences among cell lines were analyzed by one-way analysis of variance followed by Tukey’s post hoc test (α = 0.05).

RESULTS

Identification of myostatin alternative splicing forms in various tissues of avian species. Alternative splicing of avian MSTN gene was analyzed by PCR in various tissues including fat, pectoralis major muscle (PM), thigh muscle (TM), heart (H), liver (Li), lung (Lu), and kidney (K). MSTN-A was used as a normalization gene.

MSTN-A-Myc, and MSTN-B-HA using Lipofectamine 2000 (Invitrogen) and selection after 72 h with 1 mg/ml Geneticin (G418 sulfate; Gibco) were cultured and differentiated. At 0, 2, and 4 days after differentiation for 2 days. Then, cells were lysed with RIPA buffer, and cell lysates were collected by centrifugation at 15,000 g for 10 min. To coimmunoprecipitate the target proteins, cell lysates were incubated with rabbit anti-Myc-tag antibody (1:200; Cell Signaling, Danvers, MA) or mouse anti-HA-tag antibody (1:200; Cell Signaling, Danvers, MA) alternative splicing isoforms (A to E) occurred in almost all of the tissues in chicken, turkey, and quail (Fig. 1).

To investigate the sequences and alternative splicing of avian MSTN, identification of all alternative splicing forms except for MSTN-A to E in these three avian species, but the expression patterns of the isoforms were different in each tissue. Full-length (A form) and B form of MSTN was highly expressed in muscle tissues, such as PM, TM, and heart, and in all three species. The C form was highly detectable in chicken liver, lung, and kidney, while the D form was barely detectable in all tissues. The E form was highly expressed in nonmuscle tissues. Therefore, alternative splicing of MSTN varies depending on the tissue types, which may suggest differing roles for the isoforms in their respective tissues. Further studies are required to elucidate the role of these isoforms to determine whether a dynamic interplay exists for the formation and function of various organ systems.

Myostatin alternative splicing forms have unique alternative splicing sites and could produce the different forms of myostatin proteins. To investigate the sequences and alternative splicing sites, all alternative splicing forms except for MSTN-C were analyzed by PCR in various tissues of avian species.

Table 2. GenBank accession numbers for alternative splicing isoforms of avian MSTN

<table>
<thead>
<tr>
<th>Species</th>
<th>MSTN Isoform</th>
<th>GenBank Accession No.</th>
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<tbody>
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<td>Chicken</td>
<td>MSTN-A</td>
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<tr>
<td></td>
<td>MSTN-B</td>
<td>KF721282</td>
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<tr>
<td></td>
<td>MSTN-C</td>
<td>KF721283</td>
</tr>
<tr>
<td></td>
<td>MSTN-D</td>
<td>KF721284</td>
</tr>
<tr>
<td></td>
<td>MSTN-E</td>
<td>KF721285</td>
</tr>
<tr>
<td>Turkey</td>
<td>MSTN-A</td>
<td>KF721286</td>
</tr>
<tr>
<td></td>
<td>MSTN-B</td>
<td>KF721287</td>
</tr>
<tr>
<td></td>
<td>MSTN-D</td>
<td>KF721288</td>
</tr>
<tr>
<td></td>
<td>MSTN-E</td>
<td>KF721289</td>
</tr>
<tr>
<td>Quail</td>
<td>MSTN-A</td>
<td>KF721290</td>
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<tr>
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</tr>
<tr>
<td></td>
<td>MSTN-E</td>
<td>KF721293</td>
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form were cloned and sequenced from chicken, turkey, and quail muscle tissue. The MSTN-C form was cloned and sequenced only in the chicken due to low expression in turkey and quail. All sequences were submitted to GenBank, and the accession numbers for each sequence are available in Table 2. From the sequencing results, the sizes of the PCR products amplified with a primer set binding to exon 1 and 3 of MSTN were 602, 459, 379, 305, and 228 bp for MSTN-A to E, respectively. All of the alternative splicing isoforms except for the C form had the same first splicing donor site at the end of exon 1; however, they had unique splicing acceptors (Fig. 2A). MSTN-B and D forms had different splicing acceptors inside of exon 2, while the E form spliced out exon 2 and directly jumped to exon 3. These three forms followed the conventional GT-AG alternative splicing. In contrast, the C form did not use the conventional alternative splicing sites, and splicing occurred within the first and second introns to remove exon 2.

All MSTN isoforms possessed the first exon containing the start codon; therefore, each isoform could encode a separate protein. The full-length MSTN-A coding sequence contained 1,128 nucleotides, while MSTN-B, -C, -D, and -E had 390, 381, 831, and 375 nucleotides in their coding sequences, respectively. MSTN-A could be translated into 375 amino acids (aa), and MSTN-B, -C, -D, and -E could be translated into 129, 126, 276, and 124 aa, respectively (Fig. 2B). Interestingly, MSTN-B, -C, and -E forms had an early stop codon generated by alternative splicing and produced truncated proteins that had a partial MSTN prodomain, which contained a critical binding site to inhibit MSTN activity (11) (Fig. 2B, gray box within the prodomain). MSTN-D form produced a protein, which contains the prodomain region necessary for binding and inhibiting MSTN activity; however, this form also contained a mature MSTN domain. Further analysis of avian MSTN alternative splicing isoforms with mRNA sequences col-

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Fig. 2. Model of five alternative splicing isoforms of avian MSTN. A: chicken, turkey, and quail MSTN genomic DNA structure and 5 alternative splicing isoforms. MSTN-B has a deletion of the first 143 bp in exon 2. MSTN-C has an alternating splicing donor located in the first intron and acceptor located in the second intron. MSTN-D has a deletion of the first 297 bp in exon 2. MSTN-E has a complete deletion of exon 2. The dashed lines (---) indicate removed sequences, and the solid lines (——) are the same cDNA sequence or remaining sequences after splicing. Shaded areas indicate exons. B: schematic diagram of proteins translated from avian MSTN isoforms. All proteins have the signal peptide sequences (SP) and prodomain sequences from exon 1, and the prodomain has the critical peptide for binding to MSTN (gray box within the prodomain). However, mature MSTN domains are only present in the A and D forms. The B, C, and E forms have partial prodomains and no mature MSTN domains due to premature stop codons generated by a frameshift after alternative splicing. The D form has a deletion of the COOH-terminal region of the prodomain (99 aa) after alternative splicing. Three cleavage sites of pro-MSTN are indicated with arrowheads at the top. C: expression of MSTN isoforms in different avian species. As shown here, chicken, turkey, and quail have five alternative splicing isoforms (A to E); sparrow has isoforms A (HQ589117.1), B (HQ589118.1), D (HQ589119.1), and E (HQ589120.1); starling has isoforms A (HQ589113.1), B (HQ589114.1), D (HQ589115.1), and E (HQ589116.1); and duck has isoforms A (EU336991.1), B (FJ860018.1), D (HM560621.1), and E (EU336992.1).
lected from NCBI database showed that duck, sparrow, and starling had A, B, D, and E forms but did not have the C form (Fig. 2C). Therefore, avian species commonly have these types of MSTN alternative splicing isoforms that most likely possess a conserved function.

Alternative splicing of myostatin during differentiation of primary muscle cells in vitro. Five isoforms of MSTN were also identified during the myogenic differentiation of chicken, turkey, and quail primary muscle cells in cultures (Fig. 3). The full-length MSTN-A form was the dominant isoform during the
differentiation stage of muscle cells in chicken, turkey, and quail. The MSTN-B form was the subsequent dominant isoform in chickens, whereas the MSTN-E form was the second most dominant isoform in turkeys. In quail, both MSTN-B and -E forms were the subsequent most dominant isoforms. During differentiation, the expression of MSTN-B and -E forms increased in chicken muscle cells but remained consistent in quail muscle cells. In turkey, MSTN-A form was decreased during differentiation until 36 h but increased again at 48 h. Similar to the results in vivo, changes in patterns of MSTN alternative splicing were also observed during differentiation of primary muscle cells in vitro.

MSTN-B form blocks mature myostatin-mediated suppression of cell proliferation and differentiation. To clarify the expression of endogenous MSTN and its alternative splicing forms during differentiation in quail muscle clone 7 (QM7) cell line, we performed PCR to check the endogenous MSTN expression (Fig. 4A). Similar to the avian muscle tissues in vivo, the established quail myogenic cell line also produced MSTN-A as a main form and MSTN-B as a minor alternative splicing isoform during QM7 cell differentiation, suggesting QM7 cells could serve as a reliable in vitro model to further study functionality of alternative splicing isoforms of MSTN. Because the MSTN-A and -B isoforms are abundant in avian muscle tissues and QM7 cells, QM7 stable cell lines expressing no gene (EV), MSTN-A, and MSTN-B were established to investigate the functions of these two skeletal muscle isoforms. MSTN-A and MSTN-B were tagged with Myc and HA, respectively, as there are no commercially available antibodies, and the expression of the target proteins was confirmed by immunoblotting (Fig. 4B). MSTN-A-Myc was detected at 52 kDa with Myc antibody in MSTN-A expressing cells. MSTN-B-HA was detected at 25 kDa with HA antibody in MSTN-B expressing cells (Fig. 4B), higher than predicted (~16 kDa), resulting from the glycosylation site at 71–73 aa (11). In the medium collected from MSTN-A cell culture, the 13 kDa monomer of MSTN was detected (Fig. 4C); however, the 25 kDa dimeric mature MSTN was not detected in a denaturing condition. In the medium collected from MSTN-B cell culture, the 26 kDa protein, which is the secreted MSTN-B form, was detected (Fig. 4C).

To investigate the role of MSTN-A and -B in myoblast differentiation, three cell lines underwent myogenic differentiation. At 4 days of differentiation, myoblast staining with myosin heavy chain antibodies revealed that MSTN-B cells showed the highest degree of differentiation compared with EV and MSTN-A cells (Fig. 4D). Length and diameter of the myotubes were significantly different among the cell lines at both 2 and 4 days of differentiation (Fig. 5, A and B). MSTN-B cells had longer and wider myotubes compared with the EV and MSTN-A cells. MSTN-A cells had a similar width as EV cells. The myotube length of MSTN-A cells was relatively shorter than EV at day 2 but was similar at day 4, suggesting delayed growth in MSTN-A cells. The fusion index and nuclei number in the myotube were also significantly different among the cell lines (Fig. 5, C and D). Both parameters were highest in MSTN-B cells, while lowest in MSTN-A cells. As shown in Fig. 4D, total numbers of nuclei seem to be different during differentiation, although each culture started with the same cell numbers. Actual cell counting revealed that the density of MSTN-B cells significantly increased than the EV and MSTN-A cells at 2 and 4 days of differentiation (Fig. 5E). Collectively, these data clearly demonstrated that MSTN-A inhibited and
MSTN-B enhanced expansion of cell numbers and myogenic differentiation.

MSTN-B binds to pro-MSTN and inhibits its processing. Because MSTN-B contains a putative inhibitory domain (11) for MSTN activity, enhanced myogenesis created by MSTN-B could be a result of blocking antimyogenic activity of myostatin by direct binding of MSTN-B to MSTN or inhibiting the maturation process of MSTN protein. To investigate the interaction of MSTN-B protein, several MSTN derivatives were designed by deleting certain domains and adding tag sequences at the end of the COOH terminus (Figs. 6 and 7). The expression vectors containing full-length MSTN-A-Myc or MSTN-B-HA were transfected into QM7 cells (Fig. 6A). Western blot analysis revealed that the cells transfected with the vector containing MSTN-A-Myc had the pro-MSTN-A-Myc in the cells and secreted both pro- and mature MSTN-A-Myc that were detected in the culture medium (Fig. 6B). Cells transfected with the vector containing MSTN-B-HA resulted in detection of MSTN-B-HA in cells and media by Western blotting (Fig. 6B). Both pro-MSTN-A-Myc and MSTN-B-HA were detected using anti-Myc and anti-HA antibodies in the cell lysates of the culture cotransfected with both vectors. Interestingly, the levels of mature MSTN in the medium were dramatically reduced in the presence of MSTN-B-HA (Fig.

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**Fig. 6. Interaction of MSTN-B with pro-MSTN and inhibition of the formation of mature MSTN. A: illustration of MSTN exons and A- and B-forms. Signal peptides (SP, dark gray) and putative inhibitory domain (light gray) (11) of mature MSTN activity are translated from exon 1. The amino acids encoded by exon 2 are marked with downward diagonal. The mature MSTN domain is translated from exon 3 and marked with black. Each protein was tagged with Myc or HA tags as shown at the ends of COOH terminus. B: inhibition of mature MSTN production by MSTN-B. QM7 cells were cotransfected with two vectors among pCDNA3.1 containing no insert (empty vector, EV), MSTN-A-Myc, and MSTN-B-HA. Expression patterns of MSTN proteins were confirmed by immunoblotting with tag antibodies. The amount of mature MSTN protein was decreased in medium collected from cells cotransfected with MSTN-A and B. Asterisk indicates nonspecific bands, which were detected in all media. C: coimmunoprecipitation of MSTN-A and B proteins. After coimmunoprecipitating MSTN-B-HA protein with anti-Myc antibody or MSTN-A-Myc protein with anti-HA antibody, MSTN-B-HA (center) or MSTN-A-Myc proteins (right) was detected with anti-HA or anti-Myc antibody, respectively, by immunoblotting. The target band of MSTN-B-HA or full-length MSTN-A-Myc were detected only from the coimmunoprecipitates of cell lysates, which were coexpressed with MSTN-A-Myc and MSTN-B-HA.

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**Fig. 7. Identification of MSTN-B binding site on pro-MSTN. To identify the MSTN-B binding site on MSTN, cells were cotransfected with MSTN-B-HA and either EV, MSTN-prod-Myc (MSTN prodomain), or MSTN-dE2-Myc (second-exon deleted) (A) and EV, MSTN-B-Myc, MSTN-dE1-Myc (first-exon deleted) (B). After co-IP with Myc antibody, MSTN-B-HA was detected only in the precipitates from the cell lysates cotransfected with vectors containing the peptides sequences derived from the first exon (shown in gray).**
To determine which domain of MSTN-A is critical for the binding of MSTN-B, deletions of the mature myostatin domain (MSTN-pro-Myc) or exon 2 (MSTN-dE2-Myc) of MSTN-A were constructed and cotransfected into the cells with MSTN-B-HA vector. Western blot analysis confirmed the production of MSTN-B-HA, MSTN-pro-Myc, and MSTN-dE2-Myc (Fig. 7A, left). After co-IP with anti-Myc antibody, MSTN-B-HA was detected with anti-HA antibody only in the precipitate from the cell lysate containing both MSTN-A-Myc and MSTN-B-HA (Fig. 6C, middle). Likewise, MSTN-A was detected only in the precipitate from the cell lysate containing both MSTN-A-Myc and MSTN-B-HA by immunoblotting with anti-Myc antibody after immunoprecipitating the cell lysates containing both EV and MSTN-A-Myc or both MSTN-A-Myc and MSTN-B-HA (Fig. 6C, right). These results indicate that the MSTN-A and MSTN-B bind together.

To clearly demonstrate that the NH2-terminal domain encoded by exon 1 is the binding site of MSTN-B-HA, exon-1-deleted MSTN (MSTN-de1-Myc) and MSTN-B (MSTN-B-Myc containing exon 1) were generated and cotransfected with MSTN-B-HA for co-IP analysis. As shown in Fig. 7B, left, production of MSTN-HA, MSTN-B-Myc, and MSTN-de1-Myc proteins was confirmed by Western blot analysis. After co-IP with anti-Myc antibody, MSTN-B-HA was detected by immunoblotting with anti-HA antibody only in the protein lysate from the cells that were cotransfected with MSTN-B-Myc vector, but not in the lysate from cotransfection with MSTN-de1-Myc vector (Fig. 7B, right). These results clearly showed that MSTN-B binds to the domain translated from exon 1 of MSTN, and this domain is important for the interaction between full-length MSTN and MSTN-B. All these results are illustrated in Fig. 8 and suggest that MSTN-B binds to MSTN prodomain in the cell and inhibits the proteolytic processing of pro-MSTN.

DISCUSSION

Alternative splicing occurs frequently in mammals and increases the biodiversity of proteins from the limited protein-coding genes (12). About 95% of human genes that contain multiple exons are alternatively spliced (17). In the current study, we identified five alternatively spliced isoforms of avian MSTN expressed in various tissues, including skeletal muscle. The alternative splicing events of avian MSTN occurred at the common GT-AG alternative splicing sites except for MSTN-C. Early formation of stop codons by frameshift mutations was observed in MSTN-B, -C, and -E forms. Each isoform had the potential to be translated into a protein by inclusion of the start codon in exon 1. In general, pro-MSTN is composed with two major domains, propeptide domain in the NH2-terminal region and mature MSTN domain in the COOH-terminal region, which are separated after a proteolytic cleavage by furin protease at the RXXR site located between them (15, 23). Owing to an early stop codon, MSTN-B, -C, and -E forms are translated into truncated proteins that lack the COOH-terminal region of the propeptide domain and the entire COOH-terminal mature MSTN domain. However, these remaining partial propeptides derived from MSTN-B, -C, and -E forms contain the amino acid residues critical for inhibiting the mature MSTN activity (11).

It is well known that the COOH-terminal MSTN sequences form dimers, called mature MSTN, to produce its biological functions (14). There are two major proteolytic events required for generating mature MSTN. Aforementioned, the pro-MSTN protein is cleaved at the RXXR site by furin protease to generate the latent MSTN complex in which the mature MSTN is noncovalently associated with propeptides. However, this latent complex is not biologically active until the COOH-terminal mature MSTN domains are freed from the propeptides. To achieve this, the cleavage of propeptides between Arg-75 and Asp-76 by the bone morphogenetic protein-1/tolloid family of metalloproteinases dissociates the latent MSTN complex and releases the active mature MSTN domain (26). The truncated MSTN-B, -C, and -E forms are smaller than 266 amino acids of the normal propeptide region, and do

Fig. 8. Illustration of the inhibitory role of MSTN-B on pro-MSTN (MSTN-A) processing. MSTN-B binds to the NH2-terminal domain of pro-MSTN and may inhibit the formation of pro-MSTN dimers or the latent MSTN complex. These MSTN complexes could not generate the mature MSTN dimer. The MSTN prodomain, the putative inhibitory binding domain, and the mature MSTN domain are labeled with light gray, medium gray, and black, respectively.
not contain the RXRX proteolytic cleavage site. All three of these isoforms possess a critical inhibitory domain to bind pro-MSTN, which would result in the inhibition of processing to mature MSTN. In the case of the MSTN-D form, it has partial propeptide and mature MSTN domains and two proteolytic sites of MSTN. However, it is not certain whether mature MSTN formation can be achieved with the truncated propeptides, as the NH$_2$-terminal propeptide is required for the formation of proper folding and function of the COOH-terminal dimers (23, 26).

We developed two quail MSTN vectors, full-length MSTN-A and MSTN-B lacking the COOH-terminal half of the prodomain and the entire mature MSTN domain, for further exploring the functions in muscle growth and development. Because of the fact that MSTN-B was the second most abundantly expressed isoform in muscle tissues, this isoform was used for further study. As expected, overexpression of full-length MSTN-A reduced total cell number and inhibited differentiation. However, MSTN-B promoted both expansion of cell number and differentiation as evidenced by wider and longer myotubes, higher fusion rate, higher number of nuclei per myotube, and higher cell density. It indicates that MSTN-B counters the inhibitory role of mature MSTN. In the immunoblotting assay of cell lysates and medium, mature MSTN was detected only in the medium, which is suggestive that mature MSTN is generated outside of the cells. Interestingly, the production of mature MSTN was reduced in the medium of cells cotransfected with MSTN-A and MSTN-B, which suggests that MSTN-B inhibits the processing and generation of mature MSTN. It has been reported that MSTN is predominantly secreted into the extracellular matrix as a latent complex in muscle (1, 30). Free mature myostatin peptide then suppresses myoblast proliferation and differentiation by inhibiting cell cycle progression from G1 to S phase (18, 24). Therefore, increased proliferation and differentiation of the MSTN-B-transfected cells may be due to reduced concentrations of mature MSTN by MSTN-B. Characterizing an alternative splicing variant of MSTN as an inhibitor of processing to mature MSTN is a unique mechanism that is potentially relevant for regulating muscle development in avian species.

According to the binding study, MSTN-A and -B proteins interact together. Interestingly, only full-length MSTN-A was detected on the immunoblot after coimmunoprecipitation with MSTN-B-HA by using the anti-HA antibody (Fig. 6C). Although we expected that MSTN-B binds to mature MSTN, the mature form was not detected in the blot after the same co-IP with MSTN-B-HA. Further studies identified that peptide sequences translated from exon 1 were important for binding of MSTN-A and MSTN-B, but the mature MSTN domain was not involved in this binding. MSTN-B, which encodes part of the propeptide, may interrupt proteolytic processing of pro-MSTN by furin protease to generate mature MSTN by binding at a specific sequence. Therefore, this interruption leads to reduced concentrations of mature MSTN and lifts negative regulation on muscle development as shown by enhanced myoblast numbers and myotube formation.

Evolutionary divergence is evident in alternative splicing of MSTN. As stated previously, avians have at least four isoforms of MSTN with variable expression in different tissues. Rainbow trout and other salmonoids possibly possess two MSTN genes that arose from gene duplication, and these may be alternatively spliced (5). Three MSTN alternative splicing isoforms are present in the blackback land crab with one isoform exhibiting a conserved function to full-length MSTN in vertebrates, and the other two produce truncated peptides (4). Our search in GenBank and UCSC Blat leads to the speculation of alternative splicing in pigs with one isoform producing a truncated peptide devoid of the mature MSTN domain. This short form of MSTN may have similar functionality as MSTN-B in avians, but further investigation is required.

Conclusively, avian MSTN has several alternative splicing isoforms, and they are expressed depending on the tissue types. In skeletal muscle, MSTN-A and MSTN-B are the major isoforms that are potentially important for regulating muscle development. As previously stated, the MSTN-B sequence possesses a premature stop codon that translates into the NH$_2$-terminal portion of the propeptide and can bind to pro-MSTN in this region. Binding of MSTN-B to pro-MSTN inhibits the processing of mature MSTN, resulting in an increase of myoblast proliferation and myotube formation. Regulation of MSTN alternative splicing is important for muscle development in avians to control the degree of growth at various ages, and these isoforms have been evolutionarily conserved as evidenced by their presence in both domestic and undomesticated species.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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