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OSTEOPONTIN IS LINKED WITH AKT, FOXO1, AND MYOSTATIN IN SKELETAL MUSCLE CELLS

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ABSTRACT: Introduction: Osteopontin (OPN) polymorphisms are associated with muscle size and modify disease progression in Duchenne muscular dystrophy (DMD). We hypothesized that OPN may share a molecular network with myostatin (MSTN). Methods: Studies were conducted in the golden retriever (GRMD) and mdx mouse models of DMD. Follow-up in-vitro studies were employed in myogenic cells and the mdx mouse treated with recombinant mouse (rm) or human (Hu) OPN protein. Results: OPN was increased and MSTN was decreased and levels correlated inversely in GRMD hypertrophied muscle. RM-OPN treatment led to induced AKT1 and FoxO1 phosphorylation, microRNA-486 modulation, and decreased MSTN. An AKT1 inhibitor blocked these effects, whereas an RGD-mutant OPN protein and an RGDs blocking peptide showed similar effects to the AKT inhibitor. RM-OPN induced myotube hypertrophy and minimal Feret diameter in mdx muscle. Discussion: OPN may interact with AKT1/MSTN/FoxO1 to modify normal and dystrophic muscle.

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Osteopontin (OPN; SPP1) is a multifunctional cytokine with diverse functions. Its primary structure includes an arginine–glycine–aspartic acid (RGD) site that mediates interactions with the cell surface integrins (ITGs) α₁β₁, α₁β₃, and α₉β₇. Proteolytic cleavage by thrombin exposes a human SVVYGLR, ITG-binding motif, expanding the ITG-binding repertoire to include α₁β₁, α₁β₇, and α₉β₇. whereas a heparin-binding domain allows OPN to bind to CD44. OPN also has important roles in cancer progression and inflammation.

Germane to OPN’s role in muscle, a promoter polymorphism (rs28357094) alters transcription factor binding and baseline gene transcription in multiple cell types. The rs28357094 genotype was associated with an increase in biceps muscle size in women but not men, in keeping with an effect of estrogens on OPN expression. In healthy human muscle, OPN expression increased with acute mechanical loading, further suggesting a role of OPN in muscle injury and hypertrophic remodeling. The same rs28357094 polymorphism tracked with loss of muscle strength, motor function, and independent ambulation in 3 separate cohorts of dystrophin-deficient Duchenne muscular dystrophy (DMD) patients. Although not detectable in normal human or mouse muscle, OPN is highly expressed in DMD patient muscle, as well as serum and muscle of dystrophin-deficient mdx mice and golden retriever muscular dystrophy (GRMD) dogs.

In vitro, treating C2C12 myoblasts with soluble OPN protein increased proliferation and decreased fusion and migration, whereas insoluble OPN protein promoted adhesion and fusion. Given the associations of OPN gene polymorphisms, protein levels with muscle size, and its effects in vitro, we studied relationships between OPN protein and myostatin (MSTN), a known regulator of muscle mass.
METHODS

Animals. All dogs and mice were used and cared for according to principles outlined in the National Research Council’s “Guide for the Care and Use of Laboratory Animals.” All efforts were made to minimize animal suffering. Dogs were housed either at the University of Missouri (Institutional Animal Care and Use Committee No. 2435) or the University of North Carolina at Chapel Hill (Institutional Animal Care and Use Committee No. 06-338.0). GRMD dogs were identified as described elsewhere.28 Tibiotarsal (TTJ) joint angle, TTJ joint extensor and flexor tetanic torque, and cranial sartorius (CS) circumference were assessed in all dogs at 6 months of age when phenotypic results best correlate.29–33 Muscle biopsies were taken at surgery or necropsy, as previously described.34 We also utilized a murine muscle regeneration series from previously published studies.35,36 Finally, X-linked muscular dystrophy (mdx) mice were housed at the Children’s National Medical Center. At 3 weeks of age, 4 female mdx mice were injected with recombinant mouse osteopontin (rmOPN)/green dye cocktail intramuscularly into the tibialis anterior (TA) muscle of 1 limb and an equal volume of 1× phosphate-buffered saline (PBS)/green dye cocktail in the contralateral limb. Green dye was used to determine the location of the injection cocktail with microscopy. Mice were necropsied and muscle tissue was harvested.

Cell Culture. The well-established cell line, H-2Kb-tsA58 wild-type (WT), conditionally immortalized murine myoblasts,37,38 were grown in complete growth medium consisting of Dulbecco’s modified Eagle medium (DMEM), 2% L-glutamine (Gibco, Carlsbad, California), 1% penicillin and streptomycin (PAA, Dartmouth, Massachusetts), 2% chick embryo extract (Sera Lab, UK), and interferon-gamma (20 units/ml) supplemented with 20% fetal calf serum. Myoblasts were maintained at 33°C (95% air, 5% CO2) as proliferative cells at low densities in complete growth medium. To differentiate myoblasts into myotubes, cells were incubated in DMEM spiked with 1% penicillin/streptomycin, 2% L-glutamine, and 2% horse serum. Myotubes were allowed to differentiate for 4 or 5 days at 37°C (95% air, 5% CO2) as proliferative cells at low densities in complete growth medium. To differentiate myoblasts into myotubes, cells were incubated in DMEM spiked with 1% penicillin/streptomycin, 2% L-glutamine, and 2% horse serum. Myotubes were allowed to differentiate for 4 or 5 days at 37°C (95% air, 5% CO2) as proliferative cells at low densities in complete growth medium. To differentiate myoblasts into myotubes, cells were incubated in DMEM spiked with 1% penicillin/streptomycin, 2% L-glutamine, and 2% horse serum. Myotubes were allowed to differentiate for 4 or 5 days at 37°C (95% air, 5% CO2) as proliferative cells at low densities in complete growth medium.

FIGURE 1. OPN and MSTN were inversely correlated in GRMD dogs. (A) OPN and MSTN mRNA expression were inversely correlated in the CS at 4–9 weeks in GRMD dogs ($r = -0.85$, $r^2 = 0.72$, $P < 0.05$; $n = 8$), where myofiber hypertrophy is observed before gross hypertrophy. (B) OPN was inversely correlated with CS muscle circumference in GRMD dogs at 6 months of age ($r = -0.83$, $r^2 = 0.69$; $P < 0.05$; $n = 8$). (C) Cardiotoxin-induced muscle injury in WT mice resulted in an immediate and substantial increase in OPN with a temporally concurrent reduction in MSTN expression at day 1 postinjection. OPN levels eventually returned to day 0 (pre-injection) levels while MSTN returned to subnormal levels.
and/or myotubes in a 6-, 12-, or 24-well dish in serum-free DMEM. Myoblasts were incubated with rmOPN for 24 and 48 hours. AKT inhibitor #124005 \[1L6-hydroxymethyl-chiroinositol-2-(R)-2-O-methyl-3-O-octadecyl-sn-glycerocarbonate; Calbiochem/EMD4 Biosciences, Darmstadt, Germany\] was diluted in dimethylsulfoxide and 5 \( \mu \)mol/L (target IC\(_{50}\) to inhibit AKT) was added to individual rmOPN-treated wells. Myotubes were treated with 10 \( \mu \)g/ml rmOPN (optimal concentration observed in treated myoblasts) at the end of day 4 of differentiation and incubated for 24 hours until the end of day 5 to evaluate MSTN expression. To evaluate hypertrophy, myotubes were differentiated for at least 3 days, then treated for 24–48 hours with 10 \( \mu \)g/ml of rmOPN.

Human (Hu) WT OPN and Hu-RGD–KAE OPN proteins were a generous gift from Dr. Larry Fisher and were prepared as previously described.\(^{17,39}\) In Hu-RGD–KAE OPN, the RGD amino acids were mutated to lysine (K), alanine (A), and glutamic acid (E), respectively.\(^{40,41}\) For human OPN experiments, \( H-2K^{b}\)-tsA58 myoblasts were treated with 10 \( \mu \)g/ml of Hu-WT OPN or Hu-RGD–KAE OPN for 24 hours in serum-free growth medium. A peptide that blocks the biological adhesion epitope Arg-Gly-Asp-Ser \( (\text{RGDS}; \text{R&D Systems; Minneapolis, Minnesota}) \) was added at 0.05 \( (0.25 \times) \) and 0.2 \( (1 \times) \) \( \mu \)g/ml to myotubes co-treated with rmOPN for 24 hours.

Methods for light microscopy, RNA extraction, muscle regeneration time series, total protein and DNA analysis, quantitative reverse transcript–polymerase chain reaction (RT-PCR), protein isolation and quantification, Western blot, and enzyme-linked immunosorbent assay can be found in the Supplementary Material available online.

**RESULTS**

**OPN Was Correlated with MSTN and Muscle Size.** Quantitative RT-PCR data from the GRMD CS muscle at 4–9 weeks (with cellular hypertrophy evident but before gross hypertrophy)\(^{17,34}\) showed a strong negative correlation between OPN (increased) and MSTN (decreased) mRNAs (Fig. 1A). OPN mRNA levels showed an inverse correlation with CS muscle size in GRMD dogs at 6 months (Fig. 1B). OPN levels in the GRMD CS muscle at 6 months correlated positively with TTJ
angle and tetanic extensor force ($P < 0.05$; $r > 0.85$) and inversely with tetanic flexor force ($P < 0.05$; $r > -0.79$) (data not shown). We queried a previously performed murine muscle regeneration time series, which showed a dramatic increase in OPN at day 1 after cardiotoxin intramuscular injection (Fig. 1C). Interestingly, MSTN decreased during the same time period. OPN levels eventually returned to day 0 (pre-injection) levels, whereas MSTN returned to subnormal levels.

**rmOPN Protein–Treated Cells Had Decreased and Increased MSTN and AKT1 Phosphorylation, Respectively.** *H-2k*-tsA58 WT myoblasts treated with rmOPN showed a dose-dependent decrease in MSTN mRNA and protein after 24 (Fig. 2A and B) and 48 (Fig. 2C) hours of incubation. MSTN protein levels also decreased in myotubes treated with rmOPN (Fig. 2D).

Consistent with activation of the AKT1 pathway, rmOPN-treated cells had increased phosphorylated AKT1 (serine 473) levels after 24 hours of treatment (Fig. 3A and B). Exposure of *H-2k*-tsA58 myoblasts to an AKT kinase inhibitor (#124005) blocked both rmOPN-mediated AKT1 phosphorylation (Fig. 3A and B) and downregulation of MSTN at the mRNA and protein level (Fig. 4A and B).

**Recombinant Mouse OPN-Treated Cells Showed FoxO1 Phosphorylation and miRNA-486 Expression.** Addition of rmOPN to myoblast cultures increased FoxO1 phosphorylation at serine 256 (when normalized to total FoxO1) compared with control (Fig. 3A and C). This effect was blocked by AKT inhibitor #124005 (Fig. 3A and C). Intriguingly, levels of FoxO1 mRNA and protein were decreased by a fold change of −1.3 in rmOPN-treated myogenic cultures and restored by AKT inhibitor #124005 (Fig. 4A). After treating myoblasts with rmOPN, we observed a 2-fold increase in miRNA-486, a known regulator of FoxO1 and the AKT1/MSTN pathway (Fig. 5A).42,43

**OPN-Induced Reduction of MSTN Occurred through Both RGD and Non-RGD Receptors.** Myoblasts were treated with a human recombinant OPN protein with the ITG-binding RGD sequence mutated to
KAE (RGD→KAE), which partially blocked both the effects on AKT1 phosphorylation (Fig. 5B) and reduced MSTN protein (Fig. 5C). Treatment of myoblasts with Hu-WT OPN (normal RGD sequence) resulted in more profound AKT1 phosphorylation and decreased MSTN protein expression compared with Hu-RGD→KAE OPN and rmOPN (Fig. 5B and C). These results were further supported by pretreating myotubes with an RGDS blocking peptide to prevent rmOPN from binding to the RGD amino acid sequence on ITGs. The RGDS blocking peptide partially ablated the effects of rmOPN on MSTN protein expression, similar to the Hu-RGD→KAE OPN experiments, but it was not dose-dependent (Fig. 5D).

Recombinant Mouse OPN Treatment Led to Myotube and Myofiber Hypertrophy. We found an increase in myotube hypertrophy in several myotubes in rmOPN-treated cells compared with PBS control-treated cells (Fig. 6A and B). Total protein content, normalized to total DNA content, was increased after similarly treating myotubes with rmOPN for 48 hours; there was no difference in total DNA content between control and rmOPN-treated myotubes (Fig. 6C). To test our hypothesis in vivo, 3-week-old female mdx mice were co-injected intramuscularly into the TA muscle with rmOPN and a green dye cocktail, which led to an increase in minimal Feret myofiber diameter 1 week later (Fig. 6D).

**DISCUSSION**

In addition to its well-established roles in cancer progression and inflammatory states, OPN has been increasingly associated with muscle development and remodeling. A single-nucleotide polymorphism in the OPN promoter region tracked with differential muscle size in healthy women, whereas OPN knockout mice had smaller TA muscles.\(^44\) We previously showed that OPN polymorphisms were associated with muscle size in healthy women\(^13\) and hypothesized that MSTN, a well-known negative regulator of muscle mass\(^,45,46\) may share a molecular network with OPN. Similarly, we showed that CS muscle in dystrophin-deficient dogs had marked hypertrophy by 6 months of age, with sizes up to 300% of that in normal dogs.\(^32\) After finding a strong inverse correlation between OPN and MSTN in GRMD CS muscle, we hypothesized that OPN could indeed modify the MSTN muscle growth pathway. Surprisingly, we found that OPN levels were inversely correlated with GRMD CS muscle size by 6 months of age. We hypothesize that OPN exerted its down-stream effects on MSTN at 4–9 weeks of age in GRMD dogs, leading to muscle hypertrophy by 6 months of age, with a concomitant reduction of OPN at the same time. Because OPN was inversely correlated with CS muscle size at 6 months, it was no surprise to see OPN track with other functional outcome measures, such as TTJ angle and muscle strength, in the GRMD dogs, as seen in other studies.\(^13,17,34\)

We further postulated that OPN could reduce MSTN expression, which was tested in vitro. H-2k\(^2\)-tsA58 WT cells were treated with recombinant OPN proteins, and signaling pathways through ITGs/CD44, AKT1, FoxO1, and MSTN were assessed. We observed AKT1 phosphorylation (serine 473) in OPN-treated cells and a decrease in endogenous MSTN mRNA and protein. Therefore, it was no surprise to observe decreased AKT1 phosphorylation and restored MSTN mRNA and protein after co-treating cells with rmOPN and an AKT inhibitor. It should be noted that Morissette et al.\(^,47\) found that MSTN regulated AKT1-mediated hypertrophy in myotubes.\(^37\) In our study, we showed that AKT1 could indeed regulate MSTN expression, suggesting a potential feedback mechanism between MSTN and AKT1.
Humans (SVVYGLR) and mice (SLAYGR) share common OPN-binding sites in ITG and CD44 receptors. To determine the relative roles these receptors play in muscle, we treated the murine H-2kb-tsA58 WT myoblasts with Hu-WT OPN (normal RGD sequence, binding to both ITG-dependent and non-ITG receptors) and also a mutant Hu-RGD-KAE OPN (mutated RGD, binding to non–ITG-dependent receptors such as CD44). Interestingly, the Hu-WT OPN protein produced more profound AKT1 phosphorylation when compared with rmOPN treatment and Hu-RGD-KAE OPN. Hu-WT OPN increased AKT phosphorylation compared with 1× PBS control, with the latter showing the greatest effect (P < 0.05). Both human OPN proteins showed greater AKT1 phosphorylation abilities compared with rmOPN (P < 0.01). All OPN proteins increased FoxO1 phosphorylation. Hu-WT OPN, Hu-RGD-KAE OPN, and rmOPN all decreased MSTN compared with 1× PBS control (P < 0.05). Hu-WT OPN decreased MSTN protein slightly further compared with Hu-RGD-KAE OPN (P < 0.05). rmOPN decreased MSTN protein compared with 1× PBS (P < 0.05). This effect was partially blocked when rmOPN was co-treated with an RGDS amino acid blocking peptide [0.05 (0.25×) and 0.2 mg/ml (1×)] compared with rmOPN alone, but was not dose-dependent (P < 0.01).

Collectively, these data suggest that OPN treatment may signal through RGD- and non–RGD-dependent receptors, resulting in decrease MSTN. The use of human OPN not only helped determine whether RGD-dependent ITGs are involved, but also whether non-RGD receptors, such as CD44 or α5β1, contribute. One concern relates to whether bioactive growth factors made by the myeloma cell line during the generation of the rmOPN protein (R&D Systems) could confound our results. However, a reduction of MSTN expression was confirmed by the use of our Hu-WT and RGD-KAE OPN proteins generated in human marrow stromal fibroblasts. Future

FIGURE 5. AKT1 is activated by OPN through RGD- and non–RGD-dependent receptors. Hu-WT OPN (normal RGD sequence) and Hu-RGD-KAE OPN (lacking the ITG-binding RGD sequence) were tested in H-2kb-tsA58 WT myoblasts. (A) miR-486 was increased in rmOPN-treated myoblasts (FC = +2.07; P < 0.05) (* P < 0.05; ** P < 0.01). (B) rmOPN, Hu-RGD—KAE OPN, and Hu-WT OPN increased AKT phosphorylation compared with 1× PBS control, with the latter showing the greatest effect (P < 0.05). Both human OPN proteins showed greater AKT1 phosphorylation abilities compared with rmOPN (P < 0.01). All OPN proteins increased FoxO1 phosphorylation. (C) Hu-WT OPN, Hu-RGD—KAE OPN, and rmOPN all decreased MSTN compared with 1× PBS control (P < 0.05). Hu-WT OPN decreased MSTN protein slightly further compared with Hu-RGD—KAE OPN (P < 0.05). (D) rmOPN decreased MSTN protein compared with 1× PBS (P < 0.05). This effect was partially blocked when rmOPN was co-treated with an RGDS amino acid blocking peptide [0.05 (0.25×) and 0.2 mg/ml (1×)] compared with rmOPN alone, but was not dose-dependent (P < 0.01).
experiments beyond the scope of this study will be required to fully delineate the OPN–ITG binding partners.

Earlier studies have revealed that FoxO1 is a transcriptional regulator of MSTN, binding its pro-moter region to activate transcription. AKT1-mediated phosphorylation prevented FoxO1 translocation to the nucleus, thereby interfering with its transcriptional functions. We hypothesized that OPN treatment could therefore lead to both AKT1 and FoxO1 phosphorylation with a parallel decrease in MSTN expression. We indeed observed reduced endogenous FoxO1 and increased FoxO1 phosphorylation, with a parallel decrease in MSTN expression. We observed a decrease in FoxO1 mRNA after rmOPN treatment of the myoblasts and hypothesized that miRNAs targeting the mRNA were being upregulated by rmOPN treatment. Consistent with this hypothesis, miR-486, which has previously been shown to decrease FoxO1 levels and regulate the MSTN/AKT pathway, was increased 24-fold in our rmOPN-treated myoblasts. Therefore, in addition to activating the AKT pathway, OPN also appears to be associated with downstream miRs to modulate FoxO1.

After defining the molecular pathways involved, a functional relationship of OPN treatment was demonstrated with myotube hypertrophy and increased total protein content. OPN treatment was previously shown to increase myoblast proliferation, but reduce fusion. On the other hand, MSTN was observed to regulate myoblast proliferation in separate studies. Although we did not measure myoblast proliferation in the current study, we can infer that a reduction in MSTN expression may result in myoblast proliferation via OPN treatment, leading to myotube hypertrophy. Nevertheless, we observed increased minimal Feret diameter in OPN-injected mdx muscle. We hypothesized that the 3-week age group, an age with profound degeneration and regeneration within mdx skeletal muscle, would have regenerating myofibers expressing more ITG and non–ITG-dependent

**FIGURE 6.** Myotubes and muscle treated with rmOPN displayed hypertrophy. Myotubes were treated with rmOPN for 24–48 hours and subsequently evaluated on day 5 of differentiation. A 10-μg/ml dose for rmOPN optimal and was used for each experiment. (A) Myotubes were treated with 1× PBS to serve as a control. (B) Several of the H-2kb-tsA58 WT myotubes exhibited increased cell diameter (white lines) compared with 1× PBS. (C) Treatment of myotubes with rmOPN increased total protein content by 26.6% after normalizing to total DNA content compared with control (*P < 0.05). All experiments were performed in sextuplicates. (D) There was increased minimal Feret diameter in rmOPN-injected tibialis anterior (TA) muscles of 4-week-old mdx mice compared with contralateral saline-injected TA muscles. Mice were injected at 3 weeks of age (1-week treatment duration). The minimal Feret diameter was measured only for myofibers with green dye immediately adjacent to the myofiber membrane. N = 4 limbs per group.
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