PH20 Inhibits TGFβ1-Induced Differentiation of Perimysial Orbital Fibroblasts via Hyaluronan-CD44 Pathway in Thyroid-Associated Ophthalmopathy

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PURPOSE. Hyaluronan (HA) is a potential regulator of TGFβ1-induced differentiation in perimysial orbital fibroblasts (pOFs). Our study aimed to explore the effects of PH20 (a hyaluronidase) and HA on TGFβ1-induced differentiation in pOFs cultured from thyroid-associated ophthalmopathy (TAO) and control subjects.

METHODS. TAO and control pOFs (passages 3–6) were incubated with TGFβ1 ± PH20 or TGFβ1 ± HA for 72 hours and processed for various analyses, including HA electrophoresis; zSMA immunofluorescence; and quantification of zSMA (encoded by ACTA2), CD44 (a cell surface HA receptor), and SMAD2/3 (signaling molecule in the TGFβ1 pathway).

RESULTS. TGFβ1 induced myogenic differentiation (marked by zSMA upregulation) of pOFs. After TGFβ1 treatment, more HA accumulated in the TAO group than in the control group. PH20 mainly digested medium to small HA and increased the percentage of high molecular weight HA (HMW-HA) in total HA. Both PH20 and HMW-HA inhibited TGFβ1-induced differentiation in the TAO group, but neither showed significant effects in the control group. CD44 level negatively correlated with ACTA2 level in the TAO group, but no correlation was detected in the control group. Both PH20 and HMW-HA upregulated CD44 expression and inhibited SMAD2/3 expression in the TAO group, and the inhibitory effects were partially reversed by CD44 blockage. CD44 level in the control group was not affected by PH20 or HMW-HA treatment, and CD44 blockage showed no significant effects on control pOF differentiation.

CONCLUSIONS. PH20 inhibits TGFβ1-induced differentiation of TAO pOFs via HA-CD44-SMAD2/3 signaling, and the HA-CD44 signaling plays a divergent role in TAO and control pOFs.

Keywords: orbital fibroblast, differentiation, PH20, hyaluronan, CD44

Thyroid-associated ophthalmopathy (TAO) is an autoimmune disorder commonly associated with Graves’ disease and can affect various orbital tissues, including extraocular muscles.1 Based on histologic studies, the typical pathologic changes in TAO extraocular muscles are hyaluronan (HA) accumulation between muscle fibers and extension of fibrous strands within the interstitial space.2,3 These pathologic changes are attributed to perimysial orbital fibroblasts (pOFs), a unique phenotype of orbital fibroblasts that reside within extraocular muscles.4 Previous studies showed that orbital fibroblasts from TAO subjects can secrete higher levels of HA than control fibroblasts.5 Researchers have also demonstrated that orbital fibroblasts can undergo myogenic differentiation when stimulated by transforming growth factor β1 (TGFβ1), resulting in progressive fibrosis in the TAO orbit.6 However, little is known about the interplay between HA accumulation and fibrotic progression during TAO pathogenesis.

HA, a nonsulfated linear glycosaminoglycan, is the major component of the extracellular matrix. This polymeric molecule is formed with repetitive disaccharides and shows various sizes from small oligomers to high-molecular chains. HA can participate in various biologic processes and is considered a potential regulator of fibrosis.7 Notably, the function of HA strongly correlates with its polymer size. Our previous study demonstrated that large and small HA masses played divergent roles in regulating the proliferation of TAO pOFs.8 Therefore, we postulated that HA molecules with different sizes may exert distinct effects on pOF differentiation. The function of HA varies in different cell types depending on the HA receptors. Lim et al.9 reported that HA induces COX2 expression via the CD44 receptor in TAO orbital fibroblasts, and this phenomenon was not observed in non-TAO fibroblasts. CD44 is a widely expressed cell surface HA receptor, and HA-CD44 signaling participates in fibrotic processes in many diseases.10 The function of CD44 during TAO pathogenesis remains unknown. We speculated that HA-CD44 signaling may modulate TGFβ1-induced differentiation in TAO and control pOFs.

PH20 is a testicular hyaluronidase enzyme located on the human sperm surface and inner acrosomal membrane. This enzyme favors neutral pH working conditions and is suitable for both in vitro and in vivo studies. At the cellular level, HA molecular size is closely regulated by synthetic and degradative
processes. Synthetic products of different synthases are not uniform; digestive products of different hyaluronidases also vary from small oligomers to intermediate HA fragments. As a result, hyaluronidases can regulate biologic processes via different digestive products. We predicted that PH20 may interfere with TGFβ-induced differentiation by altering the HA molecular size. To test this hypothesis, we examined the influence of PH20 on HA polymer size, explored the effects of HA size on TGFβ-induced differentiation, and compared HA-CD44 signaling between TAO and control pOFs.

METHODS

Materials

Reagents included recombinant human PH20 (Abcam, Cat# ab132258), recombinant human TGFβ1 (PeproTech, Cat# 100-21), recombinant human IGF1 (R&D Systems, Cat# 291-G1-200), high molecular weight HA (HMW-HA, >950 kDa; R&D Systems, Cat# GLR002), medium molecular weight HA (MMW-HA, 75–350 kDa; R&D Systems, Cat# GLR004), and low molecular weight HA (LMW-HA, 15–40 kDa; R&D Systems, Cat# GLR001). Primary antibodies targeted vimentin (clone GT7812; Abcam, Cat# ab184631), αSMA (polyclonal; Abcam, Cat# ab5694), CD44 (clone 2C5; R&D Systems, Cat# BBA10), SMAD2/3 (clone D7G7; CST, Cat#8685), and GAPDH (clone 14C10; CST, Cat# 2118). Validations of the primary antibodies are provided on the manufacturer’s websites or in the referenced citations. Commercial kits included the rTPCR kit (Takara, Cat# RR047), SYBR qPCR kit (Takara, Cat# RR420), ELISA kit (R&D Systems, Cat# DHYAL0), ProCol-I ELISA kit (Abcam, Cat# ab210966), and capillary Western immunoassay kit (ProteinSimple, 12-250 kDa).

Subject Recruitment

Extraocular muscle tissues were collected from six TAO patients (average CAS = 2.3) during orbital decompressive surgery and from five control subjects during strabismus surgery or orbital surgery. For TAO subjects, the inclusion criteria were TAO patients with extraocular muscle enlargement; the exclusion criteria were patients who previously received orbital radiotherapy or received effective (CAS decreased to ≤2) systemic steroid therapy within 6 months. For control subjects, the inclusion criteria were patients with concomitant strabismus or cavernous hemangioma; the exclusion criteria were patients with other orbital inflammatory diseases such as IgG4-related disease. Detailed clinical information is provided in Supplementary Table S1. Informed consent was obtained from each subject, and the study protocol was approved by the institutional review board.

Histologic Staining

The muscle tissues were fixed with neutral formalin, embedded in paraffin, and sectioned in the sagittal or horizontal plane. The slides were processed with Alcian blue/periodic acid Schiff (AB-PAS) staining. Masson trichrome staining and αSMA immunohistochemical staining. Brightfield images were taken with a microscope (Leica Microsystems; Buffalo Grove, IL, USA).

Cell Culture

Primary pOFs were isolated from extraocular muscle tissues as previously described. Passaged pOFs (passages 3–6) were plated at a density of 10^5 cells/cm^2, deprived of serum for 24 hours, and incubated with vehicle (5% vol/vol PBS) or TGFβ1 (10 ng/mL) with or without PH20 (5 ng/mL), IGF1 (10 ng/mL), different HA standards (10 μg/mL), CD44 antibody (5 μg/mL) for 72 hours in serum-free DMEM.

Morphologic Analysis

Bright-field images were taken at day 4 (at confluence) in 10 random fields using a 20-objective lens. Cells were measured along the longest longitudinal axis (long axis) and the longest perpendicular axis (short axis). The ratio between the long and short axes was analyzed to compare cell morphology.

Immunofluorescence Staining

Fibroblasts were grown on glass coverslips, fixed with 4% vol/vol paraformaldehyde, permeated with 0.3% vol/vol Triton, blocked with 3% wt/vol bovine serum albumin, and incubated with primary antibodies (1:500 anti-vimentin and 1:200 anti-αSMA) at 4°C for 12 to 16 hours. After extensive rinsing, coverslips were incubated with secondary antibodies and stained with DAPI. A confocal microscope (Leica TCS SP2; Leica Microsystems) was used for imaging.

Reverse-Transcription PCR and Electrophoresis

Total RNA was extracted with TRIzol (Invitrogen) from TAO and control pOFs and reverse-transcribed to cDNA with a rTPCR kit. Nonquantitative PCR was carried out to amplify hyaluronidase genes, including HYAL1, HYAL2, HYAL3, HYAL4, and PH20. Primers are shown in Supplementary Table S2. The amplifying products were viewed by electrophoresis on ethidium bromide-stained 1% wt/vol agarose gels.

Real-Time Quantitative PCR

Real-time quantitative PCR was performed on a real-time PCR System (ViiA 7; Applied Biosystems, Foster City, CA, USA). Primers are shown in Supplementary Table S3. The amplification efficiency was evaluated by the standard curve method, and the mRNA level was normalized to GAPDH by the –DDCT method.

Enzyme-Linked Immunosorbent Assays

Culture medium was collected and centrifuged at 2000g for 10 minutes to remove debris. The supernatant was assayed immediately according to the manufacturer’s protocol. The R&D HA ELISA kit was adapted to measure HA concentrations. Notably, the HA standards in the ELISA kit were replaced by HMW-HA (>950 kDa) to improve the accuracy. The ELISA kit (Abcam ProCol-Ir1; Cambridge, UK) was adapted to determine the ProCol-Ir1 concentration. Final results are expressed as the mean ± SD nanograms per 10^5 cells.

Hyaluronan Agarose Gel Electrophoresis

Culture medium was digested with protease and centrifuged with a centrifugal filter device (cutoff size 3 kDa, Merck Millipore) to concentrate HA. HA extracts were separated by electrophoresis on agarose gels. The gel was stained with a commercial stain (Stains-All; Sigma-Aldrich, St. Louis, MO, USA) for 48 hours, destained in distilled water for 48 hours, and photographed under white light. HA concentration was quantified by pixel counting with ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA). Peaks of
different HA sizes were quantified against HA ladders and are expressed as percentages of the total HA extracts.

**Capillary Western Immunoassay (WES)**

Protein samples were extracted from unfrozen cells with RIPA reagent, quantified by a BCA protein assay, and immunode- tected with a Western blot system (WES; ProteinSimple, San Jose, CA, USA) according to the manufacturer’s protocol. Briefly, protein samples were diluted with loading buffer (ProteinSimple) and denatured at 95°C for 5 minutes. The ladder (ProteinSimple), protein samples, primary antibodies, peroxidase-conjugated secondary antibodies and chemilumi- nescent substrates were loaded onto the Western blot system (ProteinSimple). Subsequent immunodetection was performed automatically, and the results were reported as virtual gels based on chemiluminescence signals. The relative amount of each immunoreactive band was quantified by signal intensity and normalized to the GAPDH level in the same sample. To determine the optimal loading concentration for each protein target, protein samples were diluted in a concentration gradient and stained with primary antibodies (1:10 for SMAD2/3 and 1:50 for GAPDH) in the WES system. The results were analyzed for linearity of the dilution series (Supplementary Fig. S1). The optimal concentrations were 0.5 μg/μL for zSMA, 1.0 μg/μL for CD44, and 1.0 μg/μL for SMAD2/3.

**Statistics**

Commercial statistical software (SPSS, version 19.0, IBM Corp., Armonk, NY, USA) was used. To compare data between the TAO and control groups, a 2-way ANOVA test was applied, and a Sidak test was adapted for multiple comparisons. To compare data within the TAO or control group, a 1-way ANOVA was applied, and the Sidak test was adapted for multiple comparisons. To analyze the correlation between two continuous variables, Pearson correlation and linear regression were conducted. All data are expressed as the mean ± SD. P values for 1- and 2-way ANOVA are reported in the Results section and/or figure legends; P values for multiple comparisons are reported as asterisk symbols (* as $P < 0.05$, ** as $P < 0.01$, *** as $P < 0.001$, and **** as $P < 0.0001$) in the figures. A value of $P < 0.05$ was considered statistically significant.

**RESULTS**

The pOF Morphology and zSMA Expression Differ Between the TAO and Control Groups

Based on histologic staining of extraocular muscle tissues, HA (AB-PAS staining, blue), and collagen (Masson staining, blue) were overexpressed in the TAO group (Fig. 1A). Immunostaining of zSMA (brown) displayed stronger reactivity in the TAO group than in the control group (Fig. 1A). These findings, consistent with previous studies, confirmed that aggregation of HA, collagen, and zSMA contributes to TAO myopathy.

Primary pOFS were cultured from extraocular muscle tissues. At low passages, both TAO and control pOFS were elongated (Fig. 1B). At high passages, TAO pOFS transformed into a more rounded shape (Fig. 1B). The ratio of the long/short axis decreased as cell passage increased in both the TAO and control groups, and the long/short ratio in the TAO group was significantly lower than the ratio in the control group (Fig. 1B, 2-way ANOVA, $P < 0.0001$).

The upregulation of zSMA contributes to morphologic changes in fibroblasts. We therefore measured zSMA in TAO and control pOFS. Immunofluorescence of zSMA displayed a stronger signal at passage 8 than at passage 2 in both the TAO and control groups (Fig. 1C). Quantitative PCR showed higher ACTA2 (encoding zSMA) mRNA (Fig. 1C, 4.46-fold; t-test, $P = 0.0001$) in the TAO group than in the control group. To maintain stable expression of zSMA, we used cells from passages 3 through 6 in the following experiments. Detailed information about the cell strain and passage number in each experiment is provided in Supplementary Table S4.

**TGFB1-Induced Differentiation Results in HA Accumulation in the TAO Group**

Treatment with human recombinant TGFB1 time-dependently induced zSMA expression in both the TAO and control groups (Fig. 2A). The Western blot (ProteinSimple) technique confirmed the overexpression of zSMA in TGFB1-treated pOFS compared with vehicle-treated pOFS (Supplementary Fig. S2). The zSMA upregulation represents myogenic differentiation. In the following experiments, TGFB1 was used as the stimulator for myogenic differentiation with an incubation time of 72 hours, and zSMA was adapted as the marker for TGFB1-induced differentiation.

Compared with vehicle-treated pOFS, both ProCol-I and HA (precursor of collagen; 2-way ANOVA, $P < 0.0001$) and HA (2-way ANOVA, $P < 0.0001$) accumulated in the culture medium of TGFB1-treated pOFS. The concentrations of ProCol-I (2-way ANOVA, $P = 0.0004$) and HA (2-way ANOVA, $P = 0.0002$) were significantly higher in the TAO group than in the control group (Fig. 2B). These results are in accordance with the histologic findings of the extraocular muscle tissues.

HA metabolism is regulated by HA synthases (HAS) and hyaluronidases. Among the different HAS genes, HAS2 is most highly expressed in pOFS. Among the different hyaluronidase genes, HYAL2 (hyaluronidase 2) was detectable in both TAO and control pOFS regardless of TGFB1 treatment (Fig. 2C). HYAL1, HYAL3, and HYAL4 were detectable in some individual cell strains (Supplementary Fig. S3). No PH20 was observed in any cell strain (Fig. 2C). TGFB1 treatment significantly affected transcription of HYAL2 (2-way ANOVA, $P = 0.0197$) and HAS2 (2-way ANOVA, $P = 0.0225$), resulting in lower HYAL2 (0.32-fold) and higher HAS2 (2.06-fold) in the TAO group than in the control group (Fig. 2D). The above results suggested that HA accumulation in the TAO group may result from exaggerated HA synthesis and insufficient HA degradation.

**Exogenous Hyaluronidase PH20 Inhibits TGFB1-Induced Differentiation in the TAO Group**

PH20 was adapted to facilitate HA degradation in conditions with or without IGF1 (potential candidate for TAO pathoge- nesis) incubation. In vehicle-treated pOFS, IGF1 promoted zSMA expression in the TAO group (Fig. 3A, TAO: a vs. e, b vs. f) and stimulated cells to grow larger in both the TAO (Fig. 3A, TAO: a vs. e, b vs. f) and control groups (Fig. 3A, control: a vs. e, b vs. f). IGF1 induced and/or PH20 treatment (Fig. 3A, control: c, d, g, h).

ACTA2 mRNA quantification showed similar results. In vehicle-treated pOFS, the ACTA2 level was significantly higher in the TAO group than in the control group (2-way ANOVA, $P < 0.0001$), and IGF1 treatment promoted ACTA2 transcription (1.09-fold) in the TAO group (Fig. 3C). In TGFB1-treated pOFS, PH20 inhibited ACTA2 transcription in the TAO group (Fig. 3B,
no IGF1: 0.48-fold, with IGF1: 0.54-fold) but showed no significant effects in the control group. IGF1 did not influence the ACTA2 levels in TGFβ1-treated pOFs, and no significant difference was detected between the TAO and control groups (Fig. 3C).

PH20 Increases the HMW-HA Percentage, and HMW-HA Inhibits TAO pOF Differentiation

We performed agarose gel electrophoresis (Fig. 4A and Supplementary Fig. S4) to determine the HA concentrations and HA molecular weights in TGFβ1-treated pOFs. The concentration of HA was significantly decreased by PH20 (2-way ANOVA, $P = 0.0143$), resulting in a 72.76% decrease in the TAO group and a 69.36% decrease in the control group. The molecular weight of HA (Fig. 4A, 2-way ANOVA, $P = 0.0038$) was significantly altered by PH20, resulting in an increased percentage of HMW-HA in both the TAO (33.43%–49.89%) and control (24.11%–40.91%) groups and a decreased percentage of MMW-HA (52.51%–31.57%) in the control group. No significant difference in HA molecular weight was detected between the TAO and control groups (Fig. 4A).

PH20 Inhibits TAO pOF Differentiation Via CD44

We quantified αSMA by WES to determine the role of HA (LMW-, MMW- and HMW-HA) in TGFβ1-induced differentiation (Fig. 4B, Supplementary Fig. S5). The αSMA level was significantly higher in the TAO group than in the control group (two-way ANOVA, $P = 0.0060$). In the TAO group, HMW-HA suppressed αSMA expression (0.59-fold), and other HA standards (MMW- and LMW-HA) showed no significant effects on αSMA expression. In the control group, αSMA levels were not affected by exogenous HA incubation. Based on the above results, we concluded that the inhibitory effect of PH20 on TAO pOF differentiation is partially mediated by HMW-HA signaling.

CD44 Level Negatively Correlates With ACTA2 Level in the TAO Group

We compared the CD44 (a cell surface HA receptor) mRNA levels in TGFβ1-treated pOFs between the TAO and control groups.
The CD44 level in the TAO group was significantly higher than in the control group no matter with (2-way ANOVA, $P = 0.0006$) or without (2-way ANOVA, $P = 0.0001$) IGF1 treatment (Fig. 5A). IGF1 significantly induced CD44 transcription (2-way ANOVA, $P < 0.0001$), resulting in a 4.56-fold increase in the TAO group and a 3.70-fold increase in the control group. PH20 significantly promoted CD44 transcription in the TAO group (no IGF1: 2.25-fold, with IGF1: 2.24-fold) but showed no significant effects in the control group (Fig. 5A).

Exogenous HA also affected CD44 abundance in TGFβ1-treated pOFs in both the TAO (Fig. 5B, 1-way ANOVA, $P =$...
Figure 3. Hyaluronidase PH20 (with or without IGF1 coincubation) inhibits pOF differentiation in the TAO group. (A) Immunofluorescence images (magnification ×200) revealed that IGF1 promoted α-SMA (green) expression in vehicle-treated TAO pOFs (TAO: a vs. e, b vs. f), and PH20 suppressed α-SMA expression in TGFβ1-treated TAO pOFs regardless of the presence (TAO: c vs. d) or absence (TAO: g vs. h) of IGF1 incubation. In the control group, α-SMA expression was not affected by IGF1 and/or PH20 treatment (control: a-h). (B) Quantitative PCR confirmed that PH20 inhibited ACTA2 transcription in TGFβ1-treated TAO pOFs regardless of IGF1 incubation. In the control group, ACTA2 transcription was not affected by PH20. (C) In vehicle-treated pOFs, the ACTA2 level was higher in the TAO group than in the control group (2-way ANOVA, *P < 0.0001), and IGF1 promoted ACTA2 transcription in the TAO group. In TGFβ1-treated pOFs, no significant difference was detected between the TAO and control groups, and IGF1 showed no significant effects on ACTA2 transcription.
FIGURE 4. PH20 increases the HMW-HA percentage in both the TAO and control groups, and HMW-HA inhibits pOF differentiation in the TAO group. (A) Culture medium HA was extracted and separated on agarose gel. A representative gel is shown above. Lanes 1–6: HA standards (5 μg per lane) with or without hyaluronidase digestion. Lanes 7–14: culture medium HA (at least 5 μg per lane) with or without hyaluronidase digestion. Other gels are shown in Supplementary Figure S4. Peaks of HMW-, MMW- and LMW-HA were quantified by counting pixels against HA standards. The HMW-HA percentage in total HA extracts was significantly promoted by PH20 in both the TAO and control groups. The MMW-HA percentage in the control group was suppressed by PH20. The LMW-HA percentage was not affected by PH20. (B) The WES technique was adapted to quantify PH20 Inhibits TAO pOF Differentiation Via CD44.
hydrolysis, a large HA mass is degraded at a slow rate because abundance (Fig. 5C). We quantified CD44 (Fig. 5B). In the control group, only LMW-HA affected groups. In the TAO group, HMW-HA promoted CD44 expression (0.77-fold), and the suppressive effects of HMW-HA on SMA expression (0.77-fold) were reversed by anti-CD44 in the TAO group (Fig. 6B, Supplementary Fig. S7). These results demonstrated that CD44 function differs between the TAO and control groups. However, PH20 (as well as HMW-HA) treatment divergently regulated TGFβ1-induced differentiation in TAO and control groups. Lim et al. also reported that HA can induce COX2 expression via CD44 in TAO fibroblasts, but this phenomenon was not observed in non-TAO fibroblasts. Our study confirmed that the CD44 receptor is overexpressed in TAO pOFs and that the CD44 receptor mediates HA signaling during TAO pOF differentiation. Therefore, the divergence of HA activity in the TAO and control groups is in part attributed to CD44 overexpression in TAO fibroblasts. Moreover, the CD44 receptor can be divided into a standard isoform (CD44s) and many variant isoforms (CD44v). Several researchers discovered that CD44v protects against fibrosis, while CD44s facilitates fibrosis. Further studies are needed to clarify the CD44 subtypes and their representative activities in TAO and control pOFs.

IGF1 receptor (IGF1R) is overexpressed in TAO fibroblasts, and its overexpression is believed to initiate TAO pathologic changes. According to our results, IGF1 promoted ACTA2 and CD44 transcription in both the TAO and control groups, and its promotive activity was much stronger in the TAO group. A previous study showed that orbital fibroblasts can secrete IGF1 and that IGF1 secretion is elevated in the TAO group compared with the control group. We therefore assume that activated IGF1 signaling may be responsible for the upregulation of zSMA and CD44 in TAO pOFs.

The interplay between HA and HA-binding receptors is not well understood. In our study, HMW-HA inhibited TAO pOF differentiation via the CD44-SMAD2/3 pathway, while MMW- and LMW-HA showed no significant influence on zSMA expression. Some researchers have demonstrated that HA molecules of different sizes interact with different binding

DISCUSSION

Based on kinetic studies, PH20 shows lower reactivity for large HA digestion than other hyaluronidas. During PH20 hydrolysis, a large HA mass is degraded at a slow rate because the enzyme-substrate complex is inefficiently formed between HMW-HA and PH20. In our study, PH20 decreased the HA concentration and increased the HMW-HA proportion in total HA extracts. This phenomenon is partially attributed to the kinetic character of PH20. In addition, HA molecular weight is regulated by both synthetic and hydrolytic processes. We predict that intracellular HA metabolism may be altered by PH20 and contributes to HMW-HA enrichment. Further studies are needed to elucidate the metabolic balance in pOFs with or without PH20 treatment. Large HA mass predominates in intact tissues, and this inert molecule is generally considered to have anti-inflammatory and antitumorigenic activities.24,25 Smaller units of HA are generated under pathologic conditions by reactive oxygen or enzymatic degradation.26 These small fragments can also participate in biological processes such as angiogenesis, inflammatory aggravation, tumor progression and cancer metastasis.27,28 The ratio of high-to-low HA is an important factor that determines HA function in regulating biologic processes. If the high-to-low ratio decreases, small HA can compete with large HA to bind with cell surface receptors, resulting in disruption of the pericellular HA matrix and disassembly of HA-facilitated receptor clustering in lipid rafts.29,30 In our study, PH20 treatment increased the percentage of HMW-HA in total HA extracts. The increased high-to-low ratio facilitates HMW-HA activity, and contributes to the inhibitory effects of PH20 on TAO pOF differentiation. The function of HA not only depends on polymer size, but also varies among different cell types. According to our study, the molecular weight of HA (regardless of PH20 treatment) was similar between the TAO and control groups. However, PH20 had no significant effects on HMW-HA and PH20. In our study, PH20 showed lower reactivity for large HA digestion than other hyaluronidas.22 During PH20 hydrolysis, a large HA mass is degraded at a slow rate because the enzyme-substrate complex is inefficiently formed between HMW-HA and PH20. In our study, PH20 decreased the HA concentration and increased the HMW-HA proportion in total HA extracts. This phenomenon is partially attributed to the kinetic character of PH20. In addition, HA molecular weight is regulated by both synthetic and hydrolytic processes. We predict that intracellular HA metabolism may be altered by PH20 and contributes to HMW-HA enrichment. Further studies are needed to elucidate the metabolic balance in pOFs with or without PH20 treatment.

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Both CD44 abundance and the CD44-ACTA2 relationship differ between the TAO and control groups. (A) Quantitative PCR revealed higher CD44 levels in the TAO group than in the control group regardless of the presence (2-way ANOVA, \( P = 0.0006 \)) or absence (2-way ANOVA, \( P = 0.0001 \)) of IGF1 treatment. PH20 further promoted CD44 transcription in the TAO group but showed no significant effects in the control group. (B) In the TAO group, HMW-HA promoted CD44 transcription and inhibited ACTA2 transcription. Other HA standards (MMW- and LMW-HA) showed no significant effects on CD44 and ACTA2 abundance. Pearson correlation analysis confirmed a negative relationship (\( P = 0.002 \)) between CD44 and ACTA2 mRNA levels. Each spot represents a pair of data from one cell strain, and dashed lines represent 95% confidence intervals. (C) In the control group, LMW-HA suppressed CD44 transcription and stimulated ACTA2 transcription. Other HA standards (HMW- and MMW-HA) showed no statistical effects on CD44 and ACTA2 abundance. No significant correlation was detected between the CD44 and ACTA2 mRNA levels. Each spot represents a pair of data from one cell strain, and dashed lines represent 95% confidence intervals. (D) WES results confirmed higher CD44 levels in the TAO group than in the control group (2-way ANOVA, \( P = 0.0001 \)), and HMW-HA further promoted CD44 expression in the TAO group. Representative panels are shown above, and other full panels are shown in Supplementary Figure S6.
proteins and trigger distinct signal transduction pathways.\textsuperscript{12} Other researchers proposed that HA regulates downstream signaling via facilitating (by large HA) or disrupting (by small HA) colocalization of HA receptors within membrane lipids.\textsuperscript{29,30,34,35} Further studies are needed to elucidate the interaction between HA and HA receptors.

In summary, our study used PH20 to degrade excessive HA in differentiated pOFs and confirmed its inhibitory effects on TAO pOF differentiation via the HA-CD44-SMAD2/3 signaling. These findings suggest that PH20 is a promising antifibrotic reagent for TAO treatment and offer new insights into the role of HA metabolism in regulating TAO fibrogenesis.
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References