Characteristics of Hyaluronan Metabolism During Myofibroblast Differentiation in Orbital Fibroblasts

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Received: July 30, 2024
Accepted: October 12, 2024
Published: November 6, 2024

Citation: Papp FR, Katko M, Csiki R, et al. Characteristics of hyaluronan metabolism during myofibroblast differentiation in orbital fibroblasts. *Invest Ophthalmol Vis Sci.* 2024;65(13):13. https://doi.org/10.1167/iovs.65.13.13

Purpose. To study the impact of myofibroblast differentiation (MD) on hyaluronan (HA) turnover in orbital fibroblasts (OFs) focusing on the expression of its key enzymes and their potential implications in the pathogenesis of thyroid eye disease (TED).

Methods. Primary cultures of OFs were established from tissue samples (TED OFs, $n = 4$; non-TED OFs, $n = 5$). MD was induced by TGF-β1 (5 ng/mL). Measurements were performed after 24- and 72-hour treatments. The proliferation rate was determined by 5-bromo-2′-deoxyuridine (BrdU) incorporation. HA level and size were measured using an aggrecan-based ELISA-like method and agarose gel electrophoresis, respectively. mRNA expressions of myofibroblast markers and enzymes with a role in HA metabolism were determined using real-time PCR.

Results. Upregulation of type I collagen alpha1 chain, alpha-smooth muscle actin, and fibronectin indicated that OFs underwent MD after stimulation by TGF-β. After 72 hours, proliferation of untreated cultures declined, but it remained higher in myofibroblasts. Pericellular HA content, but not HA in the supernatant of myofibroblasts, increased compared to untreated cells. TGF-β was a potent stimulator of hyaluronan synthase 1 (HAS1) expression. The expression of hyaluronidase-1 and cell migration–inducing protein (CEMIP) diminished following MD, whereas the expression of transmembrane protein 2, the regulator of HA catabolism through CEMIP, was elevated. The size distribution of HA shifted toward a high-molecular-weight form following treatment with TGF-β.

Conclusions. OFs undergoing MD are characterized by decreased HA turnover as a consequence of the inhibition of hyaluronidases and HAS1 induction. Our results suggest that hyaluronidases could be potential targets in the treatment of TED.

Keywords: thyroid eye disease, hyaluronan, myofibroblast, hyaluronidase, CEMIP

Thyroid eye disease (TED) is an autoimmune condition that affects the tissues surrounding the eyes, particularly the orbital connective tissue and extraocular muscles. It is a complex and debilitating disorder, most frequently associated with Graves' disease.[1] The underlying cause of TED is the production of autoantibodies against the thyrotropin receptors (thyroid-stimulating hormone receptors [TSHRs]), which not only are present on thyroid follicular cells but are also expressed on orbital fibroblasts (OFs).[2] Autoantibodies directed against TSHRs contribute to the activation of OFs, making them the main target cells in TED. Activated OFs secrete proinflammatory cytokines that contribute to the inflammatory process in the orbital tissues by attracting immune cells to the area, further promoting inflammation and tissue damage.[3–5]

During the course of TED, the orbital tissues are infiltrated by activated mononuclear cells, such as T lymphocytes and to a lesser extent plasmocytes, mast cells, and macrophages. Among these cells, $CD163^+$ M2-type macrophages are responsible for the increased production of TGF-β.[6] In addition to lymphocytes and macrophages, OFs also express the costimulatory CD40. Interactions between CD154 (the ligand of CD40 that is localized on T lymphocytes) and the CD40 molecule on OFs further stimulate the production of inflammatory molecules and cytokines, including TGF-β, as well as the proliferation and activation of fibroblasts.[7,8]

TGF-β plays a role in the differentiation of fibroblasts into myofibroblasts, via SMAD and p38 MAPK signaling, which is a fundamental process during wound healing and fibrosis.[9,10] Characteristics of myofibroblasts include increased expression of alpha-smooth muscle actin (αSMA), type I collagen alpha1 chain (COL1A1), and fibronectin, which confers the contractile capacity of the cells.[11] TGF-β also plays a role in autocrine signaling mechanisms that maintain the myofibroblast phenotype. During the course of TED, as a result of TGF-β–induced myofibroblast differentiation, the balance of synthesis and degradation of extracellular matrix

(ECM) components changes, as the synthesis and accumulation of proteoglycans increase.[12–14]

Hyaluronan (HA) is a major glycosaminoglycan that plays an important role in cell–ECM and cell–cell interactions. In mammalian tissues, HA is synthesized in the plasma membrane by three isoenzymes referred to as hyaluronan synthases (HAS1, HAS2, and HAS3) and degraded by hyaluronidase 1 and 2 (HYAL1 and HYAL2) and the recently described cell migration–inducing protein (CEMIP), also known as hyaluronan-binding protein (HYBID) or KIAA1199.[15,16] HYAL2 degrades larger HA molecules, and HYAL1 is responsible for the cleavage of HA into di- or tetrasaccharides. Both HYAL1 and HYAL2 are required for proper HA turnover in peripheral tissues.[17] CEMIP utilizes clathrin-mediated endocytosis to degrade HA in early endosomes,[18] thus playing a crucial role in the depolymerization of HA in human skin and synovial fibroblasts.[19] Transmembrane protein 2 (TMEM2) is another regulator of HA metabolism via reducing CEMIP-dependent HA depolymerization.[19]

After its synthesis, HA is retained as a pericellular matrix, attached to the cell surface via its synthases or through binding to surface receptors.[20] A certain amount of the synthesized HA is then released from this pericellular coat via cleavage by hyaluronidases and incorporated as an integral component of the ECM. In TED, activated fibroblasts secrete large amounts of HA, the excessive deposition of which, due to its high water-retaining capacity, contributes significantly to the edema of the orbital tissues, which is an important factor in the disease process. This can lead to proptosis, double vision, and other eye-related symptoms.[4,15,21] Furthermore, the interaction of HA with HA receptors on immune cell surfaces is important for leukocyte infiltration into inflamed tissues.[22,23]

The details of changes in the HA metabolism of OFs in response to TGF-β in the orbital connective tissue in TED are not yet fully explored. Therefore, our aim was to investigate the impact of TGF-β–induced myofibroblast differentiation on HA turnover in OFs, focusing on the expression of its key enzymes.

Materials and Methods

Materials

Medium 199 with Earles' salts, Dulbecco's Modified Eagle's Medium (DMEM) High Glucose, penicillin/streptomycin, stable glutamine, fetal bovine serum (FBS), and Dulbecco's phosphate-buffered saline without calcium and magnesium (DPBS) were purchased from Biosera (Nuaille, France). Gibco TrypLE Express and recombinant human TGF-β1 were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Cell proliferation ELISA BrdU (colorimetric) kits were purchased from F. Hoffmann-La Roche (Basel, Switzerland). Bioline Agarose was purchased from Meridian Bioscience (Cincinnati, OH, USA). Sigma-Aldrich bromophenol, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide), dimethyl sulfoxide (DMSO), Proteinase K, Tris, bromophenol blue, sucrose, chloroform, sodium dodecyl sulfate (SDS), Stains All, and Tris–borate–EDTA (TBE) buffer were purchased from Merck KGaA (Darmstadt, Germany). The DuoSet Hyaluronan Kit and HA with different molecular weights were purchased from R&D Systems (Minneapolis, MN, USA). TRI Reagent solution was purchased from Molecular Research Center (Cincinnati, OH, USA). 2-Propanol and 96% ethanol were purchased from VWR International (Darmstadt, Germany). Applied Biosystems Nuclease-Free Water, High Capacity cDNA Reverse Transcription Kit, and TaqMan Gene Expression Assays were purchased from Thermo Fisher Scientific.

Tissue Samples and Cell Cultures

After informed consent was obtained from the patients according to the study protocol approved by the Regional and Institutional Research Ethics Committee of the University of Debrecen (ID: 5913/2012/EKU [84/13]), orbital connective tissue explants were acquired from the decompression surgeries of TED patients ($n = 4$, three females and one male; median age, 58 years; range, 44–63 years). Two of the TED patients underwent thyroidectomy before decompression surgery. During the course of the disease, all patients received thyreostatic medication, and one patient received radioiodine therapy to control hyperthyroidism. All patients received at least one 12 week course of intravenous glucocorticoid, and one patient received orbital irradiation. At least 1 year had passed between orbital surgery and completion of the glucocorticoid treatment or irradiation. Median time since the onset of TED was 3 years (range, 1–8). In all patients, both adipose tissue and extraocular muscles were affected to varying degrees. All patients were in the inactive phase of TED at the time of surgery (clinical activity score ≤ 3 on the 10-item scale). Control orbital connective tissues were obtained during other ophthalmic surgeries (enucleation) for non-orbital eye diseases ($n = 5$, two females and three males; median age, 66 years; range, 38–82).

OFs were cultured in accordance with the description by Bahn et al.[24] The tissues were sliced into pieces measuring approximately 2 mm^2 and seeded into culture dishes containing Medium 199 with Earle's salts with 20% (v/v) FBS, stable glutamine, and penicillin/streptomycin solution. The cells were then cultured at 37°C, 5% CO_2, in a humidified incubator. Every 3 to 4 days, the medium was changed. Resulting monolayer outgrows of adherent fibroblasts were serially passaged with TrypLE Express dissociating reagent and cultured in Medium 199 with 10% (v/v) FBS under the same conditions. Cells were kept in liquid nitrogen in freezing media for later use. Experiments were performed on the cells between passages 2 and 6.

TGF-β1 Treatment

For all experiments, OFs were plated (1.56×10^4 cells/cm^2) in 24-well or 96-well plates in DMEM High Glucose supplemented with 10% (v/v) FBS and stable glutamine, which was replaced with the same medium on the following day with or without TGF-β (5 ng/mL) for an additional 24 and 72 hours.

Cell Proliferation Assay

The assay was carried out in 96-well plates per the manufacturer's instructions. Cell cultures were treated for 2 hours with a 5-bromo-2′-deoxyuridine (BrdU) solution. BrdU is a synthetic analog of thymidine, which gets incorporated into DNA in place of thymidine during DNA synthesis. The cells were then fixed for 30 minutes using FixDenat solution, followed by the addition of peroxidase-conjugated anti-BrdU antibody for 90 minutes. This was followed by

an enzymatic color reaction by the addition of 3,3′,5,5′-tetramethylbenzidine substrate for 10 minutes; the intensity of the color change is proportionate to the amount of incorporated BrdU. Finally, a DTX 880 Multimode Detector (Beckman Coulter, Brea, CA, USA) was used to detect absorbance at 450 nm (reference wavelength, 620 nm). The optical density (OD) values are reported as OD per well, with cells plated at a density of 5000 cells/well.

Metabolic Activity Assay

To evaluate the effect of applied treatments on metabolic activity, orbital fibroblasts were seeded into 96-well culture plates (5×10^3 cells/well). After treatment, cells were incubated with 0.5-mg/mL MTT solution for 4 hours at 37°C. Supernatants were carefully removed, and the remaining adherent cells were dissolved in 100 µL/well of DMSO. The more metabolically active cells present in a sample, the more formazan is produced. Absorbance of the dye was measured at 595 nm, with background subtraction at 660 nm, using a Synergy H1 Microplate Reader (Agilent Technologies, Santa Clara, CA, USA). In the MTT assay, the OD reflects the metabolic activity of the cells and is indirectly proportionate to the number of viable cells. The OD values are reported as OD per well, with cells plated at a density of 5000 cells/well.

Quantitation of HA

Orbital fibroblasts were plated in 96-well plates. After each experiment, in order to quantify the amount of HA produced and released into the media, we collected conditioned media from the treated cells directly and conducted assays using a Hyaluronan DuoSet ELISA kit (R&D Systems). To selectively measure the amount of HA retained in the pericellular coat, the cells were washed twice with DPBS to remove residual media and any unbound extracellular components, then treated with 0.05% (w/v) trypsin–EDTA solution at 37°C for 20 minutes to degrade extracellular matrix proteins and release cell-associated HA. To inactivate trypsin, FBS was added to a final concentration of 10% v/v. After centrifugation at 1000*g* for 5 minutes, HA was measured in the supernatants.[25,26]

HA Isolation

Supernatants were collected and stored at –20°C until HA isolation was performed. For protein digestion, 110 µL of 1 mg/mL Proteinase K in 20 mM Tris buffer with 1% SDS was added to 1 mL supernatant and incubated at 60°C for 4 hours. For HA precipitation, 4 mL of prechilled 96% ethanol was added and incubated at –20°C overnight. Next day, the samples were centrifuged at 14,000*g* for 10 minutes. The pellet was washed with prechilled 75% ethanol and centrifuged again at 14,000*g* for 10 minutes. Air-dried pellets were resuspended and collected in TBE buffer and heated to 100°C for 3 minutes; they were then stored in a refrigerator overnight.[27]

HA Gel Electrophoresis

We used a modified agarose gel electrophoresis method based on Cowman's technique.[28,29] Briefly, the gel was prerun for 10 hours at 40 V, then isolated HA samples and standards (HA solutions with different molecular weights) were loaded in bromophenol blue loading buffer (0.02% bromophenol with 2-M sucrose in TBE) and run in 1.5 % agarose gel in TBE buffer (Tris–boric acid–EDTA) for 0.5 hour at constant 20 V and then for 3.5 hours at constant 40 V. Immediately after the run, the gel was placed into 30% ethanol for 1 hour. The gel was stained overnight in Stains All (0.00125 % in ethanol). Destaining the gel was performed in the dark for 30 minutes in 10% ethanol and then in water. To remove the purple background, the gel was left on the benchtop. Afterward, the gel was scanned, and densitometric analyses of the obtained images were performed using Image Studio Digits 5.2 (LI-COR Biotechnology, Bad Homburg, Germany). Summarized results were obtained using area under the curve (AUC) values of consecutive regions from the densitometric profiles of the relevant samples of each OF culture.

Table. Assay IDs of the TaqMan Assays Used

Protein	Gene	TaqMan Assay ID
Alpha-smooth muscle actin	*ACTA2*	Hs00426835_g1
Cell migration–inducing protein	*CEMIP*	Hs01552114_m1
Fibronectin	*FN*	Hs01549976_m1
Glyceraldehyde 3-phosphate dehydrogenase	*GAPDH*	Hs02786624_g1
Hyaluronan synthase 1	*HAS1*	Hs04398914_m1
Hyaluronan synthase 2	*HAS2*	Hs00193435_m1
Hyaluronan synthase 3	*HAS3*	Hs01030577_m1
Hyaluronidase 1	*HYAL1*	Hs00201046_m1
Hyaluronidase 2	*HYAL2*	Hs01117343_g1
Transmembrane protein 2	*TMEM2*	Hs00910521_m1
Type I collagen alpha 1 chain	*COL1A1*	Hs00164004_m1

Real-Time Polymerase Chain Reaction

Total RNA isolation was performed using TRI Reagent. Purified RNA samples were reverse transcribed using the High Capacity cDNA Reverse Transcription Kit. Then, the mRNA expressions of genes listed in the Table were determined using real-time PCR with TaqMan Gene Expression Assays. The reactions were carried out with the CFX Opus 96 Real-Time PCR System (Bio-Rad, Hercules, CA, USA). The ΔC_T method was used to quantify gene expression levels, and the expression of target genes was normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as the housekeeping gene.

Statistical Analysis

Statistical analysis of the research data was performed by using Prism 10 (GraphPad, Boston, MA, USA). Data are expressed as mean ± standard error of the mean (SEM). Repeated-measures analysis of variance (ANOVA) with Tukey post hoc test was performed to evaluate the differences, with TGF-β treatment as the within-subject factor and origin and the duration of TGF-β treatment as between-subject factors. The level of statistical significance was set at $P < 0.05$.

Results

Alterations in the expression of genes serving as markers of myofibroblast differentiation indicate that the fibroblast cells underwent a transdifferentiation process in response to stimulation with TGF-β (Fig. 1). The mRNA expression of

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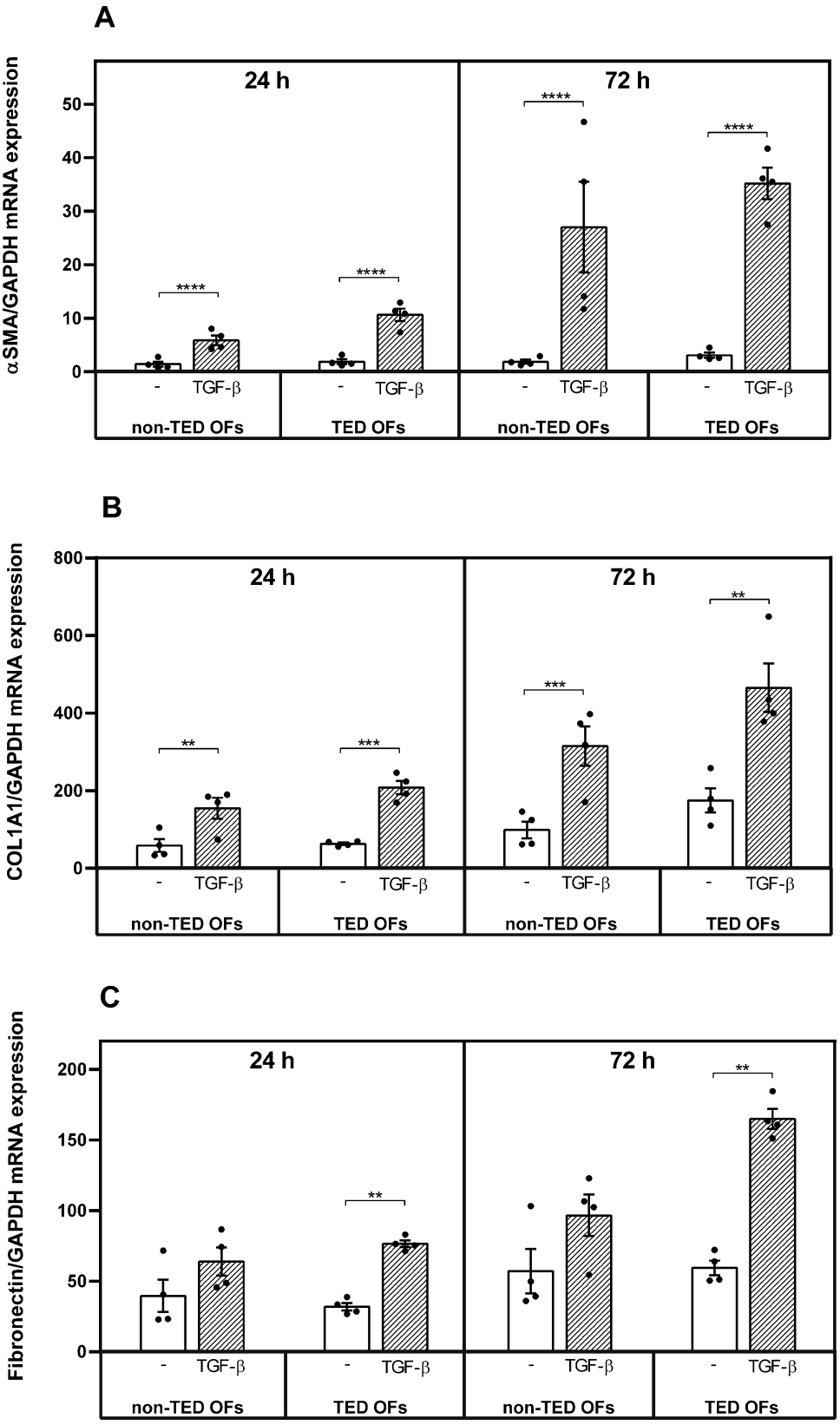

FIGURE 1. Verification of TGF-β–induced myofibroblast differentiation in OFs (non-TED OFs, $n = 4$; TED OFs, $n = 4$). (**A**) Alpha-smooth muscle actin (αSMA). (**B**) Type I collagen alpha 1 chain (COL1A1). (**C**) Fibronectin. Data are shown as mean ± SEM. *$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$.

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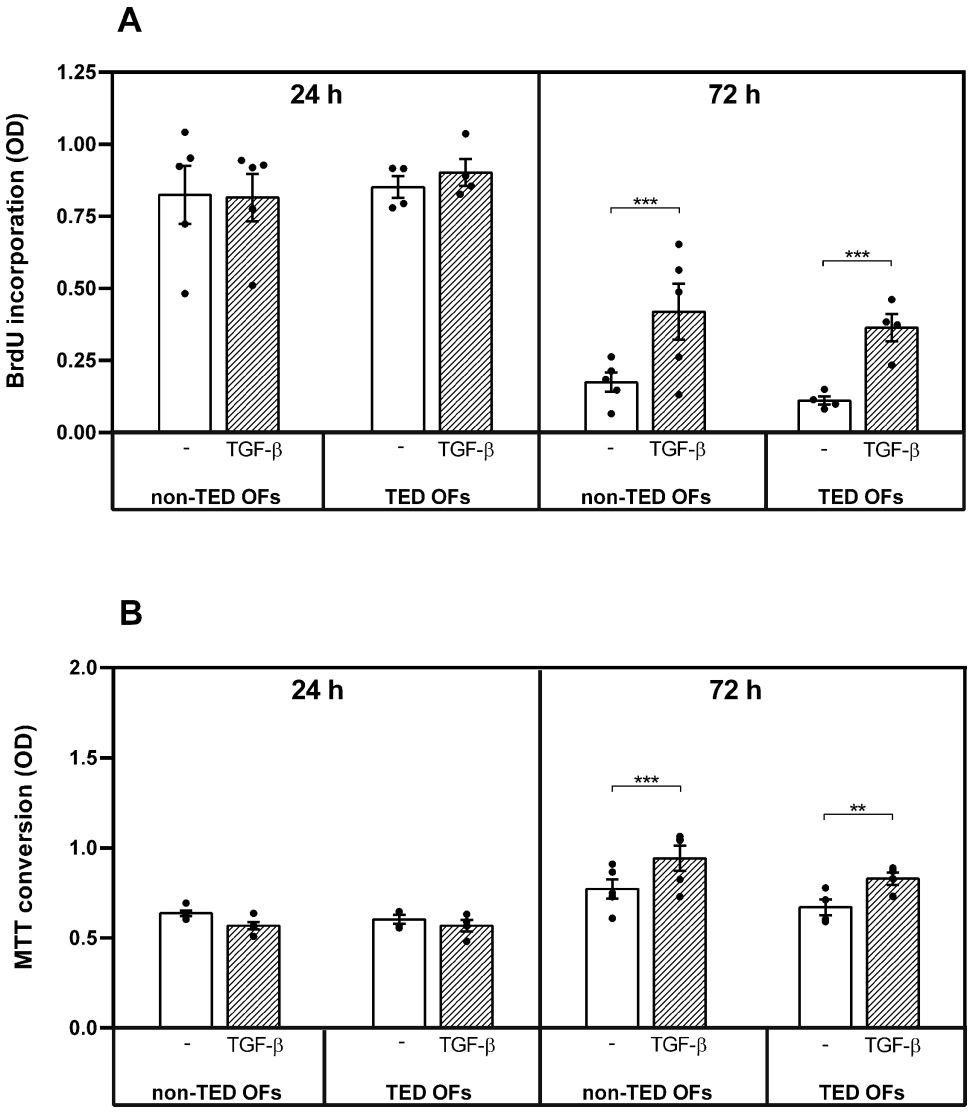

Figure 2. Proliferation rate and metabolic activity of OFs (non-TED OFs, $n = 5$; TED OFs, $n = 4$) following treatment with TGF-β for 24 and 72 hours. (**A**) Proliferation rate measured by BrdU incorporation. (**B**) Metabolic activity measured by MTT conversion. Results are presented as mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$.

αSMA and fibronectin was similar in both types of untreated OFs (TED and non-TED) at both 24 and 72 hours. TGF-β increased the αSMA expression at 24 and 72 hours in both groups (Fig. 1A). COL1A1 expression increased over time in untreated TED OFs ($P < 0.01$) and increased further after 24- and 72-hour TGF-β treatment in both non-TED and TED OFs (Fig. 1B). In TED OFs, fibronectin expression was increased during TGF-β treatment at both time points, but only a tendency to increase was observed in non-TED OFs (Fig. 1C). The effect of TGF-β on the expression of these markers was even more pronounced over time.

Treatment with TGF-β did not affect the proliferation rate of OFs at 24 hours. However, after 72 hours, despite a decrease in the baseline proliferation rates of both TED OFs ($P < 0.0001$) and non-TED OFs ($P < 0.0001$), the proliferation rates observed in TGF-β–treated cultures remained higher compared to untreated cultures (Fig. 2A). Metabolic activity of OFs did not change in the first 24 hours of TGF-β treatment; however, a modest increase was observed after 72 hours (Fig. 2B). The quantity of HA released into the media was not affected by TGF-β (Fig. 3A). However, 6.1-fold and 4.7-fold increases were detected in pericellular HA levels after 72 hours of TGF-β treatment of non-TED and TED OFs, respectively (Fig. 3B). TGF-β treatment markedly increased HAS1 mRNA expression in both TED and non-TED OFs (Fig. 4A), but no changes were observed in HAS2 and HAS3 mRNA expression (Figs. 4B, 4C).

HYAL1 expression increased with time (non-TED OFs, $P = 0.0005$; TED OFs, $P = 0.014$). This increase in HYAL1 expression was completely abolished by TGF-β–induced transdifferentiation toward the myofibroblast phenotype (Fig. 5A). TGF-β treatment did not affect HYAL2 mRNA expression (Fig. 5B).

TGF-β increased the mRNA expression of TMEM2 (Fig. 6A), whereas the expression of CEMIP decreased at both time points in OFs (Fig. 6B). The molecular weights of HA fractions appearing in the media were examined using gel electrophoresis. We found that the proportion of high-molecular-weight HA (HMW-HA) had a tendency to

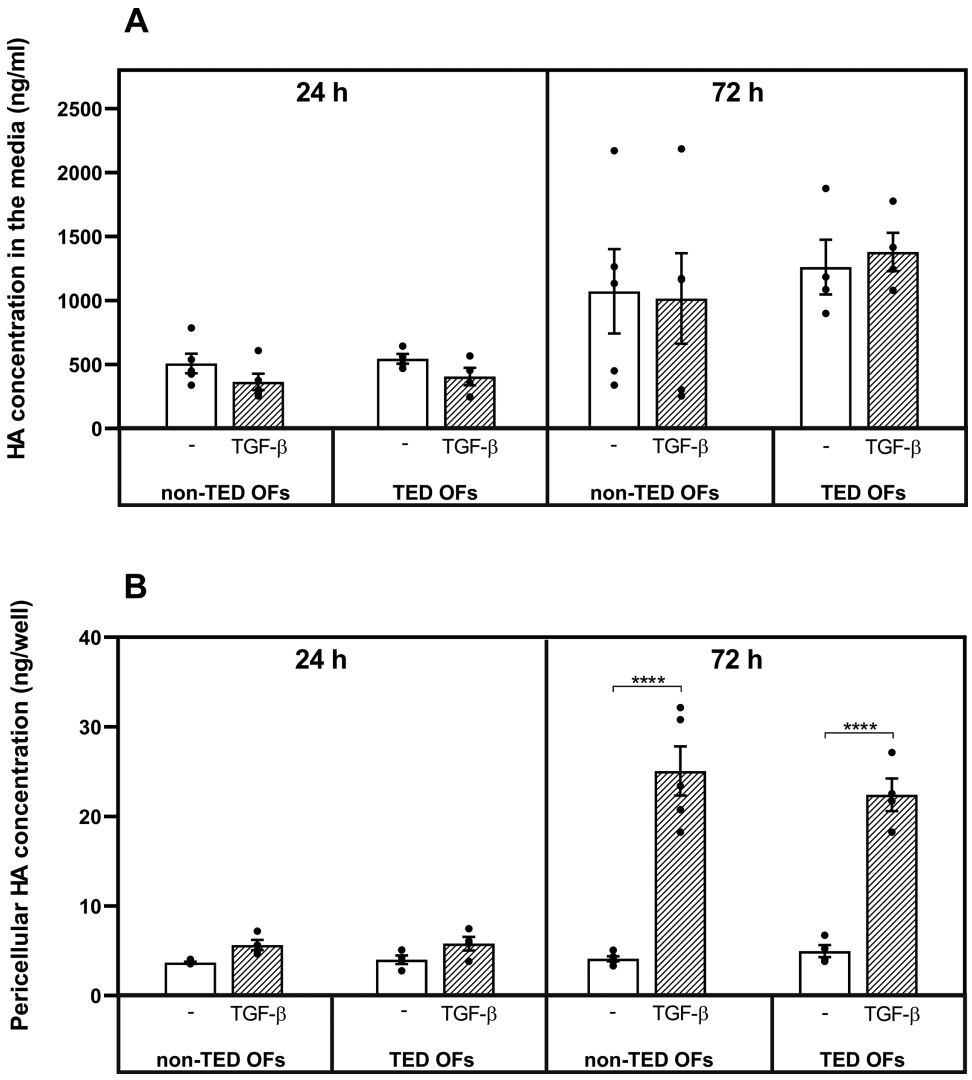

Figure 3. Effect of 24-hour and 72-hour TGF-β treatment on HA production of OFs (non-TED OFs, $n = 4$; TED OFs, $n = 4$). (**A**) HA concentration in the conditioned media. (**B**) Pericellular HA concentration. Results are presented as mean ± SEM. ****$P < 0.0001$.

increase with time and after TGF-β–induced transdifferentiation (Fig. 7).

Discussion

In the active stage of TED, the primary target cells of the autoimmune process are the OFs.[30] A major factor in their activation is the cytokines released by immune cells infiltrating the orbit. As the disease progresses, activated Thy1$^+$ OFs carry the potential to differentiate into myofibroblasts.[31,32] TGF-β, a multifunctional cytokine that is known to regulate cell growth, motility, matrix remodeling, and pericellular proteolytic activity, plays a pivotal role in both inducing this differentiation and maintaining the myofibroblast phenotype.[33] Prominent expression of TGF-β was found in the orbital connective tissue from patients with severe TED, and it was correlated with the patients' clinical activity score,[34] suggesting that the fibroblast–myofibroblast transition is present during the course of TED. Myofibroblasts are assumed to have an important role in the orbital tissue remodeling during TED,[30] some characteristics of which, including hyaluronan synthesis, have not yet been fully characterized.

TGF-β treatment is a well-established method for inducing myofibroblast differentiation in vitro. Transdifferentiation can be confirmed by the detection of the increasing expression of the stress fiber αSMA and certain ECM proteins such as COL1A1 and fibronectin. In our study, upregulation in the mRNA expression of these myofibroblast markers confirmed that specific differentiation indeed occurred after stimulation by TGF-β; this is consistent with previous findings in this field.[35–37] Although αSMA is often identified as a key marker of myofibroblasts, mesenchymal stromal cells of different origin cultured on plastic surfaces express a baseline level of αSMA, which can be further enhanced by TGF-β,[38] which was also found in both non-TED and TED OFs in our current study. The increasing αSMA expression is responsible for the contractile phenotype of myofibroblasts, which can modulate the mechanosensitive Hippo pathway by activating its effectors, Yes-associated protein (YAP), and transcriptional coactivator with PDZ-binding motif (TAZ), whereas YAP and TAZ regulate αSMA expression.[38] This

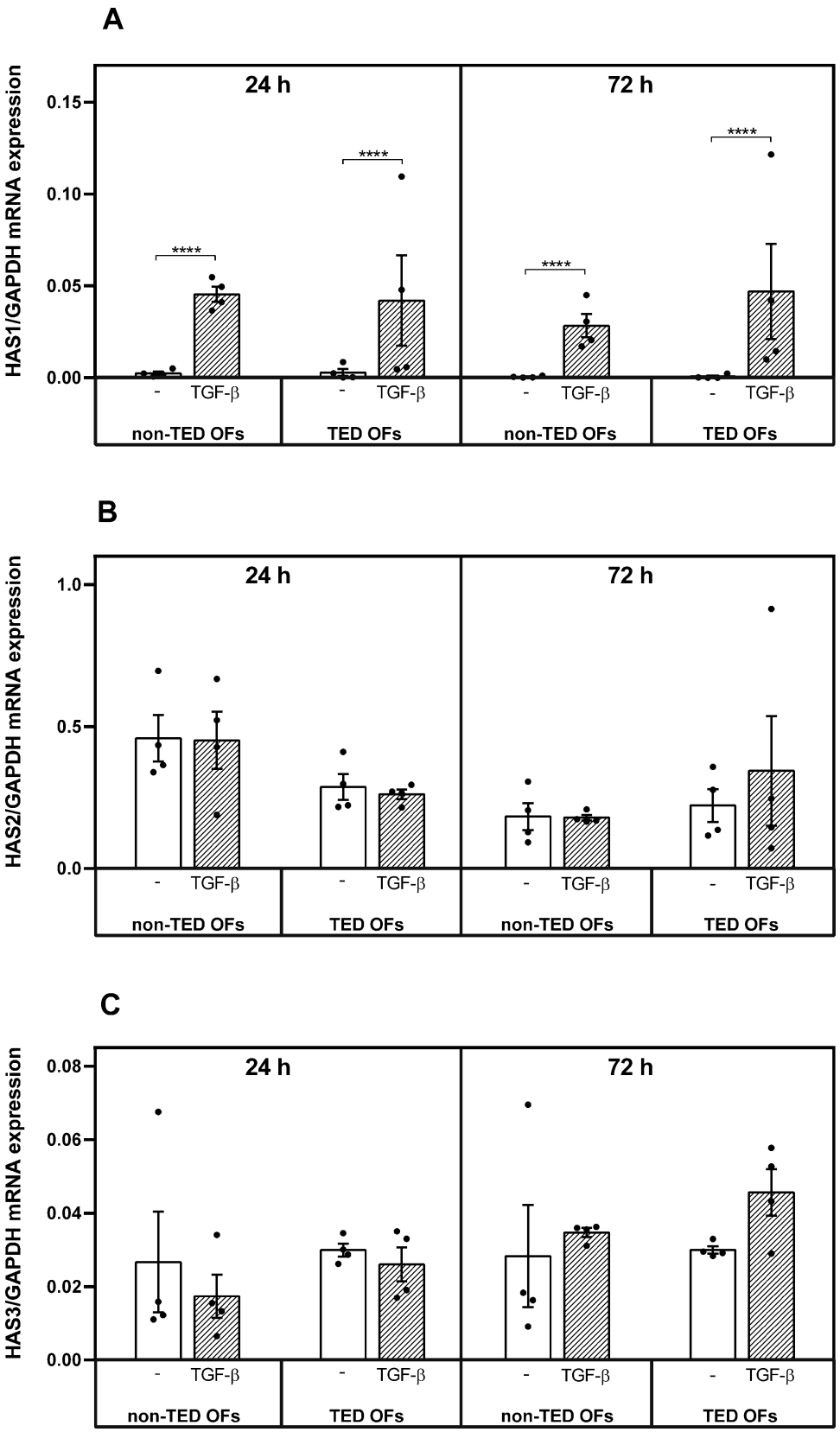

Figure 4. mRNA expression of HA synthases following treatment with TGF-β for 24 and 72 hours in OFs (non-TED OFs, $n = 4$; TED OFs, $n = 4$). (**A**) HAS1. (**B**) HAS2. (**C**) HAS3. Results are presented as mean ± SEM. ****$P < 0.0001$.

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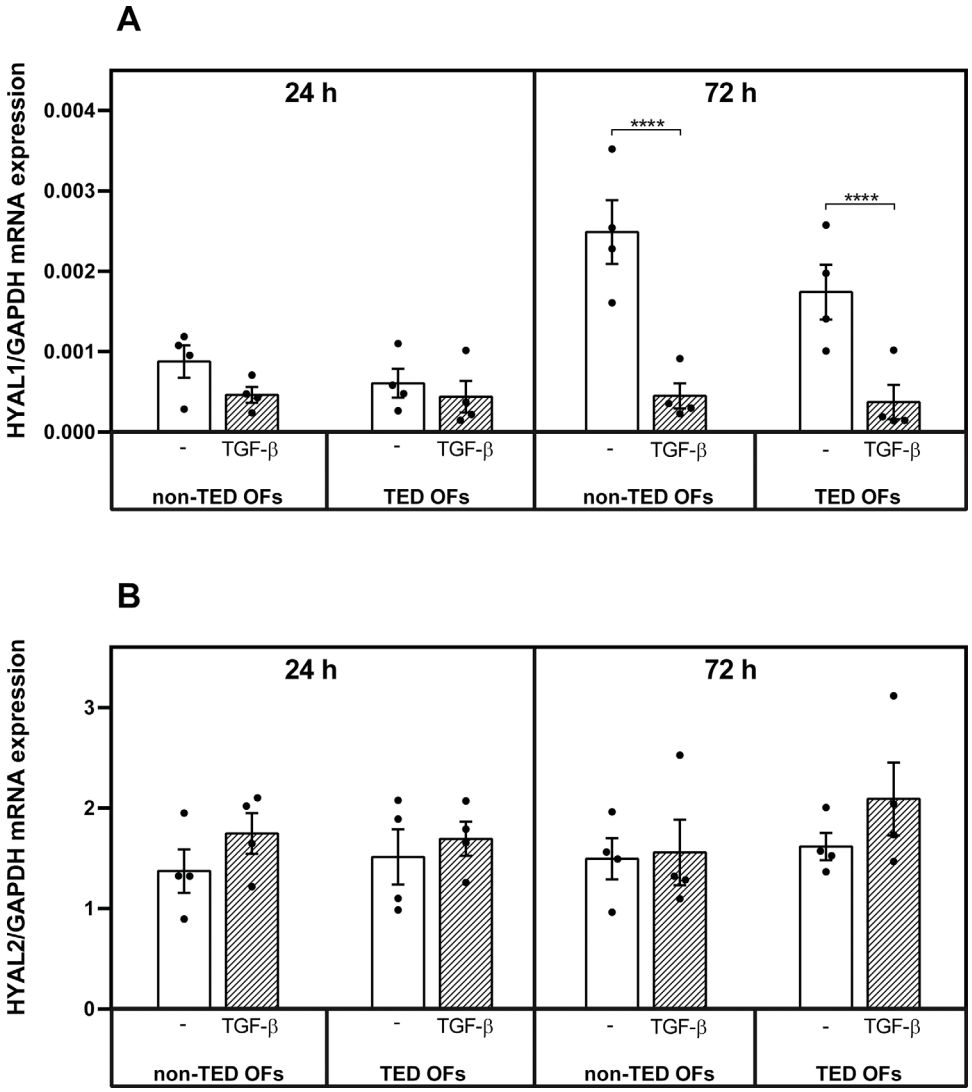

Figure 5. mRNA expression of hyaluronidases following treatment with TGF-β for 24 and 72 hours in OFs (non-TED OFs, $n = 4$; TED OFs, $n = 4$). (**A**) HYAL1. (**B**) HYAL2. Results are presented as mean ± SEM. ****$P < 0.0001$.

positive feedback loop between αSMA and YAP/TAZ can be assumed in OFs, as TGF-β elevates the expression of YAP and TAZ and their targets in OFs, whereas suppression of YAP is known to inhibit the expression of fibrotic markers including αSMA.[35]

COL1A1 is the predominant chain in the most abundant ECM protein, type I collagen. Its production by fibroblasts increases during differentiation into myofibroblasts, contributing to a denser, fibrous, collagen-rich ECM that stimulates and maintains their contractile phenotype.[39] We found high levels of COL1A1 expression in untreated OFs, which were further elevated in response to TGF-β; there was no difference between non-TED and TED OFs at the studied time points except that, in untreated TED OFs, its expression increased over time.

Fibronectin is also an ECM protein, which, along with collagens, can bind to integrins and plays an important role in regulation of cell–ECM interactions,[39] and it may act as a costimulator recognized by immune cells infiltrating orbital tissues.[40] Only TED OFs responded with a prominent rise in fibronectin expression after TGF-β treatment. The incorporation of several ECM proteins into the matrix depends on fibronectin,[41] which presumably promotes ECM remodeling in TED.

We observed that, as time progressed, there was a decrease in the baseline proliferation rates of both TED and non-TED OFs in confluent cultures, which may suggest contact inhibition as a negative regulator.[26] However, the presence of TGF-β antagonized this and ensured a certain level of proliferation at 72 hours; this finding is consistent with observations made by others[42] and was mirrored in the metabolic changes represented by MTT conversion. TGF-β has a well-documented growth inhibitory role in various cell types, but under certain circumstances, as we observed in OFs, it can also enhance proliferation.[43] The cell proliferation–stimulating effect of TGF-β is typically indirect, as it can induce the expression of growth-promoting factors.[43] In addition, HA production was found to be a major factor in the differential growth response to TGF-β in fibroblasts. Based on the study by Meran et al.,[44] we assume that high HA production by OFs contributes to their higher proliferation rate in response to TGF-β, as well as

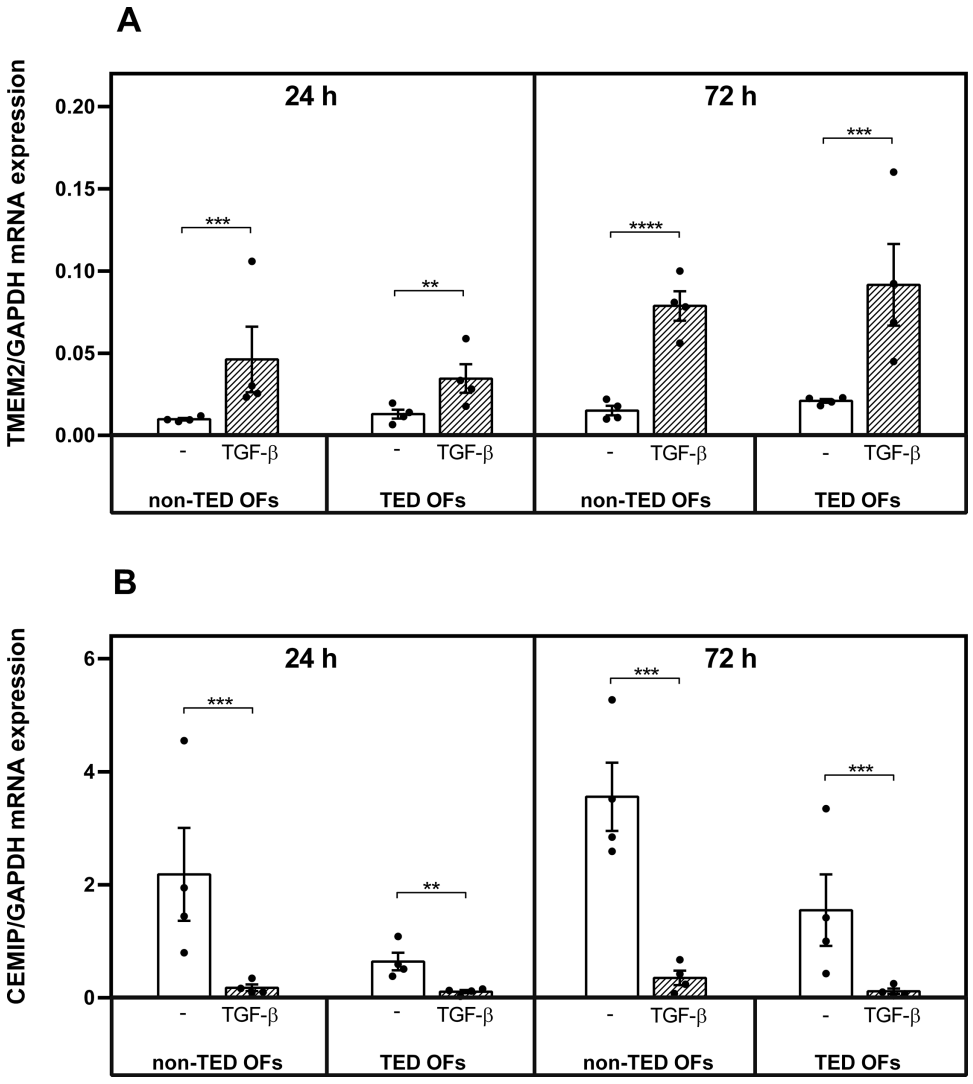

Figure 6. Expression of TMEM2 and CEMIP in response to TGF-β treatment in OFs (non-TED OFs, $n = 4$; TED OFs, $n = 4$). (**A**) TMEM2. (**B**) CEMIP. Data are shown as mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

their susceptibility to myofibroblast differentiation, which may have an important role in the pathogenesis of TED.

HA plays a crucial role in the pathogenesis of TED; due to its high fluid retaining capacity,[45] it contributes to the edematic swelling of orbital soft tissues during progression of the disease.[22] We observed a marked increase in the amount of HA in the pericellular coat of myofibroblasts after TGF-β stimulation, whereas, in the supernatant, a shift toward high-molecular-weight HA was found without a change in the HA level. Along the same lines, and in agreement with our previous studies,[26,46] HAS2 was the predominant synthase in OFs. TGF-β treatment was a potent stimulator of HAS1 expression in OFs, but HAS2 and HAS3 remained at their unstimulated levels. The contribution of HAS1 to HA synthesis substantially increased in OFs with myofibroblast phenotype. Although HAS1 has lower enzyme activity than other HA synthases, the HAS1-dependent pericellular HA coat can rapidly expand in response to cytokines,[25] suggesting that orbital myofibroblasts expressing HAS1 may achieve higher HA content in the orbital connective tissue during inflammation.

In another model system, HA accumulation during myofibroblast differentiation of lung fibroblasts was a result of reduced HA turnover rather than increased HA synthesis.[47] We found that, during TGF-β–induced transdifferentiation toward the myofibroblast phenotype, HYAL1 and CEMIP expression levels decreased, which can lead to higher HA content and matrix stabilization in the pericellular coat and may explain the shift seen toward higher molecular weight HA in the supernatant. TGF-β suppresses HA depolymerization in dermal fibroblasts via decreasing CEMIP expression while the expression of TMEM2 increases.[18] The increased TMEM2 expression seen in our in vitro model may contribute to the decreasing CEMIP expression in response to TGF-β, based on recent studies that have verified that TMEM2 facilitates the accumulation of HA by suppressing CEMIP expression.[18,19] We conclude that CEMIP and its regulator TMEM2 may have a role in HA accumulation during myofibroblast differentiation in OFs. In a mouse model, TMEM2 overexpression reduced inflammation, adipogenesis, and fibrosis in orbital tissues, suggesting that TMEM2 contributes to TED pathogenesis.[48] Because murine TMEM2, unlike human

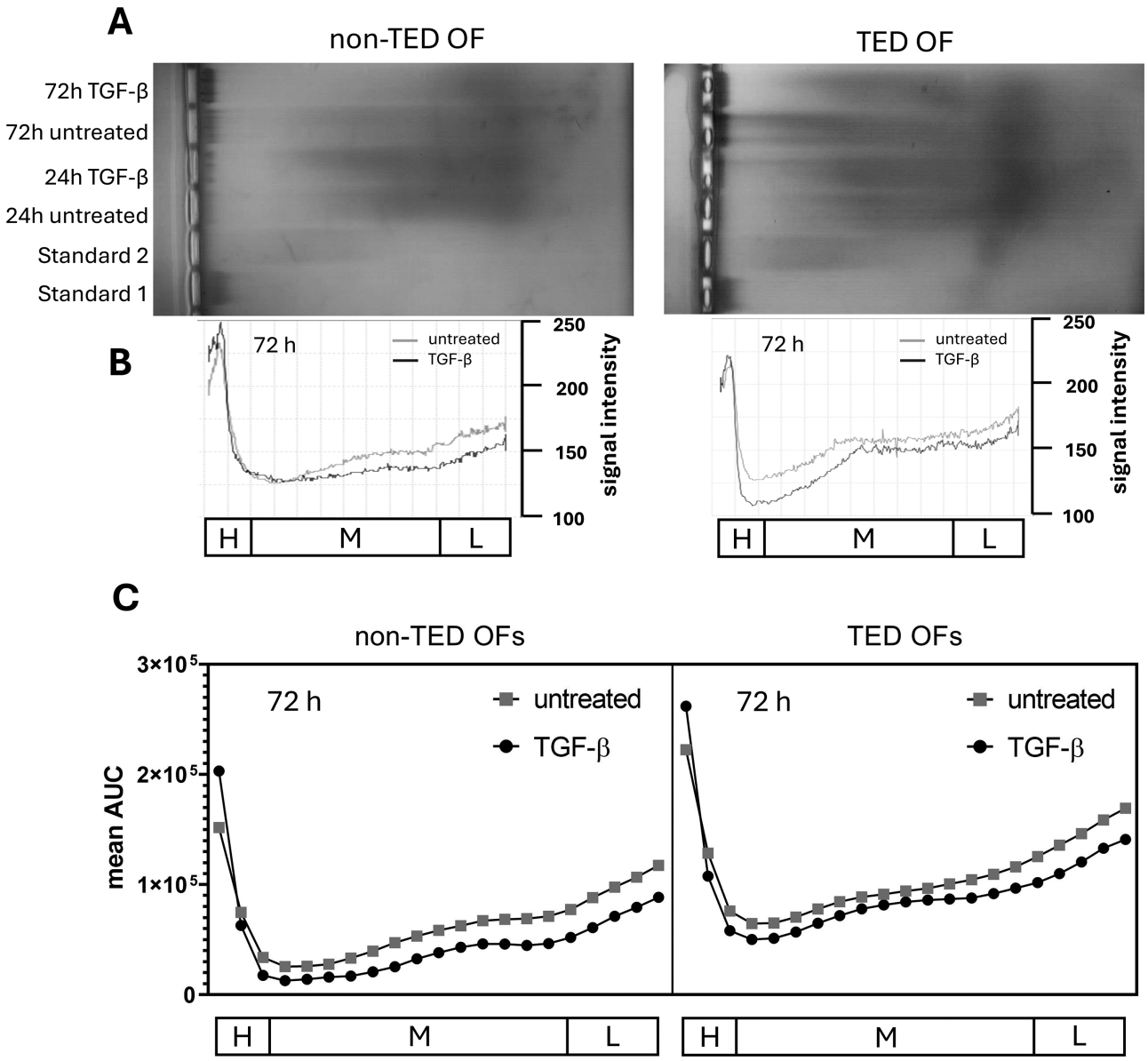

Figure 7. Results of HA gel electrophoresis for the estimation of molecular size distribution. (**A**) Representative gel electrophoresis of conditioned media of a non-TED and a TED OF culture. (**B**) Densitometric profiles of the gels. (**C**) Summarized densitometric results based on AUC values of non-TED ($n = 4$) and TED ($n = 4$) OF cultures. non-TED OF, conditioned media of orbital fibroblasts of patients without TED; TED OF, conditioned media of orbital fibroblasts of patients with TED; H, high molecular weight (HMW) range; M, medium molecular weight (MMW) range; L, low and ultra-low molecular weight (LMW and ULMW) range. Standard 1 is a mixture of HMW-HA and LMW-HA, and Standard 2 is a mixture of MMW-HA and ULMW-HA.

TMEM2,[19] possesses hyaluronidase activity, we recommend implementing human studies regarding the role of TMEM2 in TED.

In TED, the elevated expression of HAS1 and decreases in the levels of hyaluronidases in response to TGF-β impact HA turnover and lead to the accumulation of HA within the orbit, causing edema and expansion of the orbital tissues. HA accumulation may promote further fibroblast proliferation and leukocyte infiltration.[49] On the other hand, inhibiting hyaluronidases results in more HA being retained in the pericellular coat, with a simultaneous increase in the proportion of HMW-HA, which is known to have a number of beneficial properties, such as inhibiting inflammatory cell chemotaxis and phagocytosis.[50] However, during inflammation, HMW-HA can form leukocyte-adhesive HA cables and become proinflammatory.[51]

Fibroblasts derived from the orbit, whether from TED or non-TED sources, respond similarly to TGF-β treatment with regard to their HA metabolism and proliferation rates. As for myofibroblast markers, αSMA and COL1A1 increased at a similar rate regardless of the origin of the cells, whereas the increase in fibronectin expression following TGF-β treatment was significant only in TED OFs.

One of the main limitations of our experiments is that HA gel electrophoresis serves only for qualitative comparison; thus, we cannot numerically quantify the observed shift toward HMW-HA. In contrast, the quantitative HA results obtained using the HA ELISA-like method are clear but do

not provide information about the distribution of HA size fractions. Furthermore, the myofibroblast transition is only one of the complex changes occurring in TED and was examined in isolation from the broader contexts of the pathogenesis of the disease.

In conclusion, OFs that differentiate into the myofibroblast phenotype exhibit reduced hyaluronan turnover, primarily due to the decreased expression of two specific hyaluronidases. This leads to hyaluronan accumulation in the pericellular matrix, which in turn contributes to increased edema that is detrimental for the progression of TED. Based on these findings, our results suggest that targeting hyaluronidases might offer a supplementary therapeutic approach in the treatment of TED.

Acknowledgments

Supported by a grant from the Hungarian National Research, Development and Innovation Office (K143464). Project K143464 has been implemented with support provided by the Ministry of Innovation and Technology of Hungary from the National Research, Development and Innovation Fund (K_22).

Disclosure: **F.R. Papp**, None; **M. Katko**, None; **R. Csiki**, None; **E. Galgoczi**, None; **Z. Molnar**, None; **A. Erdei**, None; **M. Bodor**, None; **Z. Steiber**, None; **B. Ujhelyi**, None; **E.V. Nagy**, None

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