Hyaluronan Regulates Eyelid and Meibomian Gland Morphogenesis

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PURPOSE. The Meibomian gland (MG) produces the lipid layer of the tear film, and changes to the MG that lead to a decrease or alteration in lipid quality/content may lead to MG dysfunction, a major cause of evaporative dry eye disease with prevalence ranging from 39% to 50%. Little is known about the developmental cues that regulate MG morphogenesis and homeostasis. Our study investigates the role of hyaluronan (HA), a major extracellular matrix component, in eyelid formation and MG development and function.

METHODS. Hyaluronan synthase (Has) knockout mice were used to determine the role of HA in the eyelid and MG. Eyelids were obtained during different developmental stages and MG morphogenesis was analyzed. Tet-off H2B-GFP/K5TA mice and S-ethyl-2′-deoxyuridine (EdU) incorporation were used to determine the role of HA in maintaining slow-cycling and proliferating cells within the MG, respectively. Data were confirmed using an in vitro proliferation assay, differentiation assay and spheroid cultures.

RESULTS. Has knockout mice present precocious MG development, and adult mice present MG hyperplasia and dysmorphic MGs and eyelids, with hyperplastic growths arising from the palpebral conjunctiva. Our data show that a highly organized HA network encompasses the MG, and basal cells are embedded within this HA matrix, which supports the proliferating cells. Spheroid cultures showed that HA promotes acini formation.

CONCLUSIONS. HA plays an important role in MG and eyelid development. Our findings suggest that Has knockout mice have abnormal HA synthesis, which in turn leads to precocious and exacerbated MG morphogenesis culminating in dysmorphic eyelids and MGs.

Keywords: extracellular matrix, hyaluronan, Meibomian gland, development, stem cells

The tear film plays a vital role in maintaining a healthy ocular surface, including reducing the risk of infection. It is composed of three layers, including a lipid layer, an aqueous layer, and a mucin layer, and each layer plays a fundamental role in tear film function. The outer lipid layer is required to reduce evaporation of the remaining layers. The aqueous layer is the middle layer and is primarily responsible for lubricating the ocular surface, washing away foreign particles, and preventing infection. Finally, the mucin layer lies immediately over the cornea and helps to evenly distribute the aqueous layer and keep it adhered to the ocular surface. The lipid layer is produced by the Meibomian gland (MG), which is located within the tarsal plate in both the upper and lower eyelids. MGs contain secretory acinar cells connected to a central duct that opens onto a mucocutaneous junction at the lid margin. Macroscopically, the acinar lobules flanking the central duct are distinctly visible as yellow. The meibum is secreted by meibocytes, which are holocrine glands, becoming terminally differentiated, after which they move centripetally toward the center of an acinus and undergo degeneration and disintegration as they discharge secretions. This lifelong differentiation and destruction of acinar cells require continual turnover of the basal layer. It has been suggested that a reservoir of progenitor cells, or slow-cycling stem cells, would have to exist within MGs to provide long-term renewal of acinar cells, similar to what is seen in hair follicles and other exocrine glands, such as mammary gland, lacrimal glands, and salivary glands.

Changes to MGs that lead to a reduction or alteration in lipid quality/content may lead to a common ocular surface disorder, MG dysfunction (MGD). Alterations in the lipid content of the outer lipid layer lead to an unstable tear film and increased evaporation of the aqueous layer. MGD is a major cause of evaporative dry eye disease, with prevalence ranging from 39% to 50% in the United States. Factors that influence the development of MGD are age and gender, with an increased prevalence seen in women. The use of contact lenses in low humidity environments is also a risk factor for MGD. The use of some prescription medications such as isotretinoin and severe cases of Demodex brevis mite infestation may lead to the onset of MGD. Elucidating the mechanisms that govern healthy development and homeostasis of the MG are of vital importance to understand the pathological processes that lead to MGD.

Hyaluronan (HA) is a nonsulfated glycosaminoglycan composed entirely of repeating disaccharides of glucuronic acid and...
N-acetylglucosamine, which are alternately linked by β-1,3- and β-1,4-glycosidic bonds. HA is a ubiquitous component of the extracellular matrix (ECM) and is responsible for approximately 3% of the human dry body weight. HA plays an integral role in maintaining tissue integrity and homeostasis, development, inflammation, tissue repair, and wound healing. Alterations in HA expression have been shown to lead to age-related pathologies, such as arthritis and tumorigenesis.

We have recently shown that HA matrices are present within stem cell niches and play an important role supporting stem cells. HA is present in tissues in primarily two forms: high molecular weight HA (HMWHA) of approximately 2000 kDa and low molecular weight HA (LMWHA) of approximately 200 kDa. These two forms of HA have drastically distinct physiologic functions and, therefore, the size of the HA chains dictates the composition and function of specific HA matrices that are formed. HMWHA is primarily correlated with development, homeostasis, and tissue integrity, whereas LMWHA is primarily correlated with tissue remodeling and pathogenesis. Therefore, targeting the HA content during pathogenesis, including injury, inflammatory disorders, cardiovascular disease, and cancer, is becoming an extremely attractive strategy for intervention. HA is naturally synthesized by HA synthases (HASs), of which vertebrates have three isoforms: HAS1, HAS2, and HAS3. The mechanism by which HAS enzymes regulate the length of the growing HA chain during the biosynthetic process, which could explain the evolutionary pressure for mammals to express three HAS isoforms, remains to be established. It has been speculated that HAS1 and HAS2 produce primarily HMWHA, whereas HAS3 produces primarily LMWHA; however, some groups have shown that all HAS isoforms have the ability to make both HMWHA and LMWHA. Interestingly, naked mole rat (Heterocephalus glaber) fibroblasts have been shown to secrete extremely-high molecular weight HA (EHMHA), over five times higher than that found in other mammals including humans. This EHMHA contributes to the exceptionally high longevity and unusual resistance to cancer displayed by naked mole rats.

MGs and hair follicles share similar developmental cues. Similar to hair follicles, MGs are formed by epithelial-mesenchymal interactions that lead to the invagination of epithelial cells into the mesenchymal tissue. Intricate ECM-cell interactions regulate epithelial cell invagination and proliferation into the eyelid. Importantly, as with other skin appendages, the ECM must be digested at the tip of the developing MG to allow for its ingrowth into the mesenchymal tissue. Previous studies have demonstrated the important role HA plays in the skin development and skin appendage formation. Unpublished findings made during a previous study revealed that the tarsal plate was an HA-rich tissue.

The ECM-MG interplay that governs the development of the MG remains elusive. Given the important role HA plays in morphogenesis and the lack of information regarding the role of the ECM in MG development, we hereby investigated the role of HA in eyelid formation and MG development and function.

**Materials and Methods**

**Animal Maintenance**

**Mouse Strains and Genotyping.** Mouse models were generated to knock out the HASs: Has1 and Has3 null mice were bred to generate Has1−/−Has3−/− mice and Has2flox/flox mice were bred with K14-rtTA (stock number 008099; The Jackson Laboratory, Bar Harbor, ME, USA) and tetO-cre (stock number 006224; The Jackson Laboratory) to generate compound K14-rtTA, tetO-cre (TC), and Has2flox/flox that lack Has2 in the MG, namely Has2−/−/L-MG. K14-rtTA was selected to induce the excision of Has2 in the eyelid and MG, because MG abnormalities were noted during our previous studies using these mice. Administration of doxycycline chow was used to induce K14-driven persistent and irreversible excision of Has2 in the MG of triple-transgenic mice (K14-rtTA; Has2flox/flox, generating Has2−/−/L-MG). The mice were housed in a temperature-controlled facility with an automatic 12-hour light-dark cycle at the Animal Facility of the University of Houston. Experimental procedures for handling the mice were approved by the Institutional Animal Care and Use Committee, University of Houston. K5TA mice were kindly provided by Adam Glick and were bred with H2B-GFP mice (The Jackson Laboratory) to generate bigenic mice with green fluorescent protein (GFP) expression in keratin 5 (K5) expressing cells regulated by doxycycline administration (tet-off). H2B-GFP/K5TA mice were maintained at Heath Park Animal Facility, Cardiff University and bred under protocol 3 of project license number (PPL) 30/3022. The use of animals in experiments in the United Kingdom is regulated under the Animals (Scientific Procedures) Act 1986. All animal procedures adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The identification of each transgene allele was determined by PCR genotyping with tail DNA.

**Administration of Doxycycline to Induce Conditional Knockout of HAS2 in the MG and Induce GFP Expression in H2B-GFP/K5TA Mice**

Doxycycline chow was fed to Has2−/−/L-MG in order to induce K14-driven persistent and irreversible excision of Has2 in K14+ cells, which would include the MGs. The mice for this study were induced at embryonic day 0 (E0). Transgenic mice including the pregnant dams were fed with doxycycline chow (1 g of doxycycline/kg of chow; Custom Animal Diets LLC, Bangor, PA, USA). For such, the female mice were placed on doxycycline chow upon mating at 1 day old (Dox diet catalog no. AD3008S; Custom Animal Diets, Bangor, PA, USA) and kept on this special diet through to weaning, and, thereafter, the weaned mice were maintained on doxycycline chow. Mice lacking either the K14-rtTA or tetO-cre allele were also supplied with doxycycline chow and used as littermate controls. H2B-GFP/K5TA were pulsed from P0 to P28 to label all MG cells with nuclear GFP and then fed doxycycline for 28 days chase. K5 cells that divide in the chase label all MG cells with nuclear GFP and then fed doxycycline (2 g/kg) for 28 days chase. K5 cells that divide in the chase phase lose 50% GFP fluorescence, which means that slow-cycling epithelial progenitors can then be identified by their retention of the GFP label.

**Tissue Processing During Developmental Stages**

Mice were euthanized with CO2 and heads collected at postnatal days (PDs) 0, 1, 5, and 12, eyelids were collected at PD26 or from adult mice (7-8 weeks old), and tissues were immediately fixed in 4% paraformaldehyde at 4°C. The next day, the tissues were washed with PBS. For whole-mount staining, the eyelids were dissected and processed for whole mount. At least 15 samples were analyzed for whole-mount staining per experimental point. For histology, the eyelids were embedded in tissue freezing medium and processed for cryosectioning. At least five samples were analyzed per experimental point. Both male and female mice were used in the study, yielding comparable results.
Oil Red O Staining

The eyelids were immersed in oil red O solution (Sigma-Aldrich Corp., St. Louis, MO, USA) that stains neutral triglycerides and lipids, and were left under gentle agitation at room temperature for 1 (pup eyelids) or 2 (adult eyelids) hours, protected from light. The eyelids were then destained by gently rinsing with deionized water. The eyelids were then mounted and imaged.

Whole-Mount Staining

The eyelids or tarsal plates were incubated in permeabilization buffer (0.25% dimethyl sulfoxide [DMSO], 0.25% Triton X-100, and 15% sucrose in PBS) for 30 minutes at room temperature under constant rotation and rinsed with PBS. Thereafter, the eyelids were stained with Alexa-546 Phalloidin (Thermo Fisher Scientific, Wilmington, DE, USA) and biotinylated HA binding protein (HABP-385911; Millipore, Billerica, MA, USA) in staining buffer (0.25% DMSO, 0.01% saponin, and 15% sucrose in PBS) overnight under constant rotation at 4°C followed by incubation of the NeutrAvidin Alexa Fluor 555 conjugate (SouthernBiotech., Birmingham, AL, USA). The eyelids were washed with staining buffer (0.25% DMSO, 0.01% saponin, and 15% sucrose in PBS) overnight under constant rotation at 4°C followed by incubation of the NeutrAvidin Alexa Fluor 555 conjugate overnight under constant rotation at 4°C protected from light. The eyelids and tarsal plates were washed with staining buffer followed by PBS and mounted on glass slides in Fluoromount-G (SouthernBiotech., Birmingham, AL, USA). The eyelids were imaged using an LSM 800 confocal microscope with Axiocam 503 color (Zeiss). For analyzing the data, we carried out the 2-ΔΔCt methods for the HAS1^+/−; HAS3^+/− samples and the 2-ΔΔCt method for the HAS^+/−/+ samples by using the CXF Connect Realtime System's software and Microsoft Office Excel. The primer combination used for HAS1 was forward 5'-CTATGCTACCAAAGTATACCTCG-3' and reverse 5'-TCTTGGGAGTAAGAATGTGAC-3'; for HAS2 was forward 5'-CCTGGCTCTCTCAAATTCTACTG-3' and reverse 5'-ACAATTGCACTTTGTGCAGCTC-3'; for HAS3 was forward 5'-GATGTCCAAATCTCAACAAG-3' and reverse 5'-CCCAATTAACATGTGGTCAAGC-3'; for Actb was forward 5'-CAGTGGACATCCCAGCAAT-3' and reverse 5'-TGATACCTCCTGCTGTTGTA-3'; and for Gapdh was forward 5'-AAGGACATCTCTGGAGATCG-3' and reverse 5'-CCCTGGTGCGTCATGCTGC-3'. Mice were isolated from three HAS1^+/−; HAS3^+/− and wildtype (wt) mice and five HAS^+/−/+ mice. Mice were isolated from the left and right, upper and lower eyelids, and pooled for each individual animal. The expression profile of each individual animal was analyzed independently and presented as a single point in the graphs.

Immunohistochemistry

Eyelids were collected and immediately fixed in 4% buffered paraformaldehyde overnight and then embedded in Tissue-Tek embedding medium (Sakura Finetek USA, Inc., Torrance, CA, USA) for cryosectioning. Sections (10 μm) were cut using a Leica CM 1950 (Leica, Buffalo Grove, IL, USA) cryostat and collected on Fisherbrand SuperfrostPlus Gold microscope slides (Thermo Fisher Scientific). Upon use, sections were incubated for 30 minutes at 60°C, and excess tissue embedding medium was removed with PBS. Unspecific protein binding sites were blocked with 10% fetal bovine serum (FBS) prepared in PBS containing 0.1 M saponin. Sections were then incubated with the primary antibodies anti-Krt14 (PRB-155P; Covance, Princeton, NJ, USA) and HA binding protein (Millipore). Sections were washed and incubated with Neu- trAvidin Alexa Fluor 555 conjugate and anti-rabbit produced in donkey conjugated with Alexa 488 for 1 hour at room temperature. A secondary control was carried out with rabbit IgG isotype control (Abcam, Cambridge MA, USA) in place of the primary antibody and did not yield any staining (results not shown). Slides were mounted in Fluoromount-G and imaged under an LSM 800 confocal microscope (Zeiss).

RNA Extraction From MGs and Real-Time PCR Analysis

Eyelids were obtained from adult mice and the tarsal plate containing the MG were isolated under a stereomicroscope and snap frozen in liquid nitrogen. Total RNA was isolated from the MGs by using Trizol Reagent (Invitrogen, Carlsbad, CA, USA). Upon isolation, the RNA concentration and purity were determined using a spectrophotometer at 260 and 280 nm. First strand cDNA was reverse transcribed using 2 μg of total RNA and the high capacity cDNA Reverse Transcription kit (Catalog no. 4368814, lot 00593854; Applied Biosystems, Lithuania), according to the manufacturer’s protocol. Quantitative real-time PCR amplification was performed on 1 μl or 50 ng of the cDNA (1:5) with specific primers for Has 1-3, β-actin (Actb), and Gapdh and the kit Powerup SYBR Green Master Mix (Catalog no. A25918; Applied Biosystems) in a CFX Connect Realtime System from Bio-Rad Laboratories (Bio-rad, Hercules, CA, USA) by using the activation cycle of 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The specificity of the amplified products was analyzed through dissociation curves generated by the equipment, yielding single peaks. Negative controls were used in parallel to confirm the absence of any form of contamination in the reaction. Analysis of the data was carried out using the 2^−ΔΔCt methods for the HAS1^+/−; HAS3^+/− samples and the 2^−ΔΔCt method for the HAS^+/−/+ samples by using the CFX Connect Realtime System’s software and Microsoft Office Excel. The primer combination used for Has1 was forward 5'-CTATGCTACCAAAGTATACCTCG-3' and reverse 5'-TCTTGGGAGTAAGAATGTGAC-3'; for Has2 was forward 5'-CCTGGCTCTCTCAAATTCTACTG-3' and reverse 5'-ACAATTGCACTTTGTGCAGCTC-3'; for Has3 was forward 5'-GATGTCCAAATCTCAACAAG-3' and reverse 5'-CCCAATTAACATGTGGTCAAGC-3'; for Actb was forward 5'-CAGTGGACATCCCAGCAAT-3' and reverse 5'-TGATACCTCCTGCTGTTGTA-3'; and for Gapdh was forward 5'-AAGGACATCTCTGGAGATCG-3' and reverse 5'-CCCTGGTGCGTCATGCTGC-3'. Mice were isolated from three HAS1^+/−; HAS3^+/− and wildtype (wt) mice and five HAS^+/−/+ mice. Mice were isolated from the left and right, upper and lower eyelids, and pooled for each individual animal. The expression profile of each individual animal was analyzed independently and presented as a single point in the graphs.

In Vivo Cell Proliferation Assay

To verify whether HA regulates the proliferation of MG epithelial cells, we injected adult mice with EdU so that cells actively dividing would incorporate EdU into their newly synthesized DNA (Life Technologies, Eugene, OR, USA). Mice were labeled with 200 μg EdU/g body weight by intraperito- neal injection for 6 hours. Thereafter, the mice were euthanized by CO₂ inhalation and their eyelids collected and fixed in 4% buffered paraformaldehyde overnight. The eyelids were processed for cryosectioning or tarsal plate whole-mount staining. Sections (10 μm) were cut and collected, as mentioned above. The incorporated EdU was developed using the Click-IT Alexa Fluor 488 kit (Life Technologies, Carlsbad, CA, USA) to reveal the incorporated EdU. The sections or whole-mount eyelids were then counterstained with HA and DAPI, as described above and mounted in Fluoromount-G. Images were captured using an LSM 800 confocal microscope.

MG Epithelial Cells

Immortalized human MG epithelial cells (hMGs) were kindly provided by David Sullivan and cultured as previously...
described. In short, hMGCs were maintained in keratinocyte serum-free medium containing 5 ng/mL epidermal growth factor (EGF) and 50 μg/mL bovine pituitary extract (Invitrogen). Experiments were carried out with cells between passages 5 and 7.

**In vitro Proliferation Assay**

The effect of HA on MG cell proliferation was assayed in vitro by measuring the incorporation of 5-bromo-2-deoxyuridine (BrdU) into the newly synthesized DNA of replicating cells (BrdU Cell Proliferation Kit, EMD Millipore, Darmstadt, Germany). The hMGCs were plated at a seeding density of 5000 cells/well in 96-well plates and allowed to grow for 24 hours in the presence or absence of HA or 4-methylumbelliferone (4-MU; Sigma-Aldrich Corp.). The concentrations used were 0.02% and 0.05% of HMWHA; 0.02% and 0.05% of LMWHA; or 0.25 mM, 0.5 mM, or 2 mM of 4-MU. After 24 hours, with the cells in their exponential growth phase, BrdU was added to each well and left for 2 hours. The cells were then fixed and the detection of BrdU incorporation carried out according to the manufacturers’ instructions. Absorbance in each well was read as optical density (OD) by using a spectrophotometer microplate reader (Fluostar Omega, BMG Labtech, Offenburg, Germany) set at a test wavelength of 450 nm. Each condition was tested in triplicate and three separate experiments were carried out. The mean of each individual experiment is presented as a single point on the graph.

**Hanging Drop 3D Culture System**

In order to further study the role of HA on MG development, the 3D hanging drop culture system was used. Ten-microliter drops of hMGCs at a density of 2.5 × 10⁴ cells/mL were placed on the inside of a 60-mm petri dish lid (Eppendorf, Hauppauge, NY, USA). Various brands were tested, and the Eppendorf-treated cell culture dishes presented the highest surface tension and were, therefore, the most suitable for the hanging drop technique. The hMGCs were placed in culture in the presence or absence of HA, specifically 0.01% ULMWHA, 0.05% LMWHA and 0.05% HMWHA were used. The lids were then inverted and placed over a petri dish containing 1 mL PBS to prevent the drops from drying out. On day 4, FBS at a final concentration of 10% was added to the cells to trigger differentiation, and the cultures were maintained for a further 6 days. The cells were maintained at 37°C and 5% CO₂ for a total of 10 days after which images were taken and the spheres were fixed in 2% paraformaldehyde for 30 minutes. The spheres were washed with PBS, blocked, and stained with Alexa Fluor 488 Phalloidin (Cell Signaling, Eugene, OR, USA) and HA binding protein (Millipore) followed by NeutrAvidin Alexa Fluor 555 conjugate. Nuclei were counterstained with DAPI.

**MG Epithelial Differentiation Assay**

Previous studies have shown that the hMGCs used in this study remain progenitor-like when maintained in serum-free media. However, when FBS is added to the media, the cells terminally differentiate and start to accumulate lipid droplets within the cytoplasm. In order to verify whether HA regulates MG cell differentiation and lipid production, hMGCs were stimulated with FBS in the presence or absence of HA and lipid levels were measured using HCS LipidTOX Red neutral lipid stain (Invitrogen). The effect of HA on hMGCs differentiation was assayed by either adding HMWHA, LMWHA, or 4-MU to the media, or coating the dish with poly-D-lysine (PDL) followed by HMWHA (Lifecore Biomedical, Chaska, MN, USA) or LMWHA (Lifecore Biomedical). For assaying the effect of soluble HA, hMGCs were cultured in keratinocyte serum-free medium (Invitrogen) containing 5 ng/mL EGF and 50 μg/mL bovine pituitary extract. When cells reached approximately 70% to 80% confluence, the media was replaced with Dulbecco’s modified Eagle’s medium and Ham’s F12 (1:1) containing 10 ng/mL EGF and 10% FBS supplemented or not with 0.05% HMWHA, 0.05% LMWHA, or 0.5 Mm 4-MU. When lipid droplets were evident within the cytoplasm of the control cells, LipidTOX was added to the media and left for 24 hours, after which the cells were imaged and fixed with 4% paraformaldehyde. For assaying the effect of an HA substrate on differentiation and lipid production, culture dishes were treated with PDL (20 μg/mL) for 2 hours at room temperature, followed by 0.1% HMWHA or 0.1% LMWHA overnight at 4°C. Thereafter, MGs were seeded on the coated plates and cultured in Ham’s F12 (1:1) containing 10 ng/mL EGF and 10% FBS keratinocyte serum-free medium (Invitrogen) containing 5 ng/mL EGF and 50 μg/mL bovine pituitary extract. Controls were carried out with PDL-coated dishes and uncoated dishes. When lipid droplets were evident within the cytoplasm of the control cells, LipidTOX was added to the media and left for 24 hours, after which the cells were imaged and fixed with 4% paraformaldehyde. The experiments were carried out in sextuplicate and repeated twice. A total of 25 images were captured for each experimental group and the number and size of lipid droplets graded by two individual investigators in a blinded manner.

**Statistics**

All values are presented as standard deviation of the mean. The difference between two groups was compared by Student’s t-test and between more than two groups by 1-way ANOVA. Values with P ≤ 0.05 were considered to be statistically significant. Statistical analysis was performed using the GraphPad Prism version 5 software package (GraphPad Software, San Diego, CA, USA).

**RESULTS**

**Has Knockout Mice Present Altered MGs**

Eyelids were obtained from Has1⁻/⁻; Has3⁻/⁻, Has2⁻/⁻/AMG, and wt adult mice stained with oil red O and analyzed under a stereomicroscope (Fig. 1). The MGs of Has1⁻/⁻; Has3⁻/⁻ and Has2⁻/⁻/AMG mice were found to be enlarged, elongated, and contained a larger lipid volume when compared with wt mice (Fig. 1).

**Histologic Analysis of MGs From Has1⁻/⁻; Has3⁻/⁻, Has2⁻/⁻/AMG, and Wt Mice**

Eyelids were obtained from Has1⁻/⁻; Has3⁻/⁻, Has2⁻/⁻/AMG, and wt adult mice, processed for histology, and sagittal (Fig. 2) cryosections were prepared. The expression of HA and K14 were analyzed in the tissues (Fig. 2). K14 is an intermediate filament that is present in the cytoskeleton of cells of epithelial origin. The development of the MG relies on developmental cues triggered by epithelial-mesenchymal interactions that lead to the invagination of epithelial cells into the mesenchymal tissue. Thus, in the eyelid, epithelial cells and cells of epithelial origin, such as the MG and hair follicles, all express K14. K14 staining confirmed that Has1⁻/⁻; Has3⁻/⁻ and
HAS2Δ/MG mice present larger MGs compared with wt mice (Fig. 2). Moreover, HAS1Δ/C0/C0; HAS3Δ/C0/C0; and HAS2Δ/MG mice present dysmorphic MGs, which are no longer elongated in shape, with a larger number of meibocytes per acinus (Fig. 3A). Wt mice present an average of 12 meibocytes per acinus in a single plane, whereas HAS1Δ/C0/C0; HAS3Δ/C0/C0; and HAS2Δ/MG mice present 20. Based on the HA staining pattern of eyelids of wt mice, HA is expressed in eyelids primarily by tissues of mesenchymal origin. In Figure 2, a white dashed line was used to outline the eyelid and MG. Strong HA expression was seen circumventing MG acini, whereas low HA expression was detected within MG cells (Fig. 2). We did not observe a significant change in the HA staining pattern between eyelids of knockout mice when compared with wt mice. However, based on the HA staining, HAS1Δ/C0/C0; HAS3Δ/C0/C0; and HAS2Δ/MG mice do not present a decrease in HA expression in eyelids (Fig. 2).

**FIGURE 1.** Whole eyelids were obtained from wt, HAS1Δ/C0/C0; HAS3Δ/C0/C0, and HAS2Δ/MG adult mice, fixed, trimmed, and mounted to enable flat mount. Images were captured under a stereomicroscope (top panel). After imaging, the eyelids were stained with oil red O solution and then mounted and imaged under a stereomicroscope using white light (middle panel) and the 555 channel (lower panel). Scale bar: 250 μm.

**FIGURE 2.** HA (red) and K14 (green) staining in sagittal sections of adult eyelids from wt, HAS1Δ/C0/C0; HAS3Δ/C0/C0, and HAS2Δ/MG adult mice. Staining revealed that HA is present throughout the eyelid primarily in tissues of mesenchymal origin. In the MG, HA is present surrounding the acinar basal cells throughout the gland and also along the central collecting duct. The upper eyelid is shown in the top panel and the lower eyelid in the bottom panel. A white dashed line was used to outline the MG and the eyelid. White arrows indicate the palpebral conjunctiva. Nuclei were counterstained with DAPI (blue). Scale bar: 100 μm.
HA Distribution in the MG and Tarsal Plate

Given that HA is highly expressed surrounding the MG and tarsal plate, we investigated the organization of the HA matrices in these tissues. The distribution and organization of the HA network was analyzed in tarsal plate whole mounts of HAS1/C0/C0/C0; HAS3/C0/C0/C0, HAS2/D/MG, and wt mice. Interestingly, HA forms distinct matrices in the tarsal plate and surrounding the MB. The whole-mount analysis revealed that HA forms an intricate 3D HA network surrounding the basal cells of the MG, with geometry resembling chain-link woven netting (Fig. 4, lower panel), as previously shown.51 However, in the tarsal plate HA forms cable-like structures (Fig. 4, upper panel), as previously shown.27

HAS Knockout Mice Present Dysmorphic Eyelids With Epithelial Hyperplasia

HAS1/C0/C0/C0; HAS3/C0/C0/C0 and HAS2/D/MG mice present dysmorphic eyelids. The mice present epithelial hyperplasia leading to hyperplastic growths arising from the palpebral conjunctival epithelium, causing the development of prominent papules or nodules. The hyperplastic outgrowths were present along the palpebral conjunctival epithelium of all HAS1/C0/C0/C0; HAS3/C0/C0/C0 and HAS2/D/MG mice, leading to a loss of the smooth wiping surface of the eyelids (Fig. 2). Due to the outgrowths on the inner surface of the eyelids, HAS1/C0/C0/C0; HAS3/C0/C0/C0 mice present an increase in conjunctival surface area, including an increase in the number of goblet cells (Fig. 2, white arrow). HAS1/C0/C0/C0; HAS3/C0/C0/C0 mice present a 2-fold increase

Figure 3. The number of meibocytes and goblet cells in wt, HAS1/C0/C0/C0; HAS3/C0/C0/C0, and HAS2/D/MG adult mice. The number of meibocytes present in a single plane of an acinus of wt, HAS1/C0/C0/C0; HAS3/C0/C0/C0, and HAS2/D/MG adult mouse MGs was counted in a double blinded manner (A). The number of goblet cells present along the palpebral conjunctiva in a single plane of wt, HAS1/C0/C0/C0; HAS3/C0/C0/C0, and HAS2/D/MG adult mouse eyelids was counted in a double blinded manner (B). *P ≤ 0.05.

Figure 4. The organization of HA (red) was analyzed in wt, HAS1/C0/C0/C0; HAS3/C0/C0/C0, and HAS2/D/MG adult mouse tarsal plate whole mounts. Nuclei were counterstained with DAPI (blue). Scale bar: 10 µm.
in the number of goblet cells when compared with wt mice (Fig. 3B). Curiously, the tet-off H2B-GFP/K5tTA mice revealed that the slow-cycling epithelial cells of the palpebral conjunctiva also lie within an HA matrix (results not shown). Thus, HA could also play a role in regulating the epithelial stem cells of the palpebral conjunctiva, and changes in HA lead to an increase in cell proliferation.

**Analysis of the Expression Profile of Hass in the MGs of Has1−/−;Has3−/−, Has2<sup>+/AMG</sup>, and Wt Mice**

Due to the fact that mice lacking the HAS enzymes did not present a loss of HA surrounding the MGs, we investigated the expression profile of the different Hass to verify if Has1−/−;Has3−/− upregulates Has2 and Has2<sup>+/AMG</sup> upregulates Has1 and/or 3 through a compensatory mechanism. For such, eyelids were obtained from Has1−/−;Has3−/−, Has2<sup>+/AMG</sup>, and wt adult mice and the tarsal plates dissected for RNA extraction. The expression profiles of Has1−/− were analyzed by real-time PCR (Fig. 5). Interestingly, Has1−/− are expressed in the MB, with Has2 being the most highly expressed of the synthases and Has1 the least expressed (Fig. 5A). Has1−/−;Has3−/− mice present an upregulation of Has2 when compared with the wt mice, thereby compensating for the loss of Has1 and Has3 (Fig. 5C). The lack of Has1 and Has3 expression was confirmed in the Has1−/−;Has3−/− mice (Figs. 5B, 5D). Has2<sup>+/AMG</sup> mice presented a significant reduction of Has2 expression, to 25% of that of wt mice, and therefore confirming the loss of Has2 in the MGs of Has2<sup>+/AMG</sup> mice (Fig. 5F). Has2<sup>+/AMG</sup> mice upregulate the expression of Has1 and/or 3 in the MGs (Figs. 5E-G). Curiously, of the five mice analyzed, two Has2<sup>+/AMG</sup> mice upregulated solely Has1 expression, two mice upregulated solely Has3 expression, and one mouse upregulated both Has1 and 3 (Figs. 5E-G).

**Slow-Cycling MG Progenitor Cells Are Present Within an HA Matrix**

Putative stem cell populations have been speculated to exist within the MG, providing a constant supply of new basal cells. Due to the lack of well-characterized stem cell markers for MG stem cells we used tet-off H2B-GFP/K5tTA mice (as previously shown) to verify whether K5 label-retaining cells (LRCs), which are slow-cycling progenitor cells, are localized within the HA-rich niches. Although stem cell niches have still to be identified within the MG, slow-cycling progenitor cells (H2B-GFP<sup>+</sup> LRCs) have been shown to be present within the MG ductule that terminates at each acinus by using H2B-GFP/K5tTA mice after pulse/chase labeling. To visualize slow-cycling epithelial progenitors, eyelids were obtained from H2B-GFP/K5tTA adult mice and the tarsal plate processed for whole mount. HA staining revealed that H2B-GFP<sup>+</sup> LRCs (28-day chase) within the MG were embedded within an HA matrix (Fig. 6B). Moreover, the whole mount analysis of the tarsal plate confirmed that HA forms an intricate 3D HA network surrounding the MG acini, with geometry resembling chain-link woven netting (Fig. 6B), as previously shown.

**Role of HA During MG Morphogenesis**

To investigate how changes in HA expression could lead to altered MGs in adult Has1−/−;Has3−/− and Has2<sup>+/AMG</sup> mice, we investigated the morphology of the MGs at different developmental stages. For such, eyelids from P5, P12, and P26 mice were obtained and stained with oil red O and imaged under a stereomicroscope (Fig. 7). In Figure 7, a black dashed box has been used to outline a MG in the wt mice and an equiproporionate box placed over a MG in the Has1−/−;Has3−/− and Has2<sup>+/AMG</sup> mice of the same time-point, to aid in comparing the size of MGs between samples. At P5,
HAS1−/−;HAS3−/− mice present longer MGs when compared with wt and HAS2−/−/AMG mice (Fig. 7, top row). At P12, the MGs of HAS1−/−;HAS3−/− and HAS2−/−/AMG mice present more branching and are therefore wider than those of wt mice (Fig. 7, middle row). At P26, the MGs of HAS1−/−;HAS3−/− and HAS2−/−/AMG mice are both longer and wider than those of wt mice, to the extent that there is no gap between the MGs (Fig. 7, bottom row). Therefore, changes in HA expression lead to precocious and excessive MG development.

The role of HA in the development of MGs was also determined by whole-mount immunostaining. Eyelids of P5, P12, and P26 mice were processed for whole mount, stained with phalloidin, and imaged under a confocal microscope evidencing the structure of the MGs (Figs. 8 and 9, MGs are outlined with a white dashed line). The precocious formation of the MGs can be clearly seen in the eyelids of the mice at P5 (Fig. 8). The MGs of wt mice at P5 are cylindrical and do not present any signs of branching at this developmental stage (Fig. 8). On the other hand, both the HAS1−/−;HAS3−/− and HAS2−/−/AMG mice already present branching of the MGs at P5 (Fig. 8). Both the increase in length and width of the MGs at P12 and P26 of HAS−/−;HAS3−/− and HAS2−/−/AMG mice compared with wt mice can also be evidenced by phalloidin staining (Fig. 9). As seen with the images captured with oil red O staining, phalloidin staining evidences that HAS1−/−;HAS3−/− and HAS2−/−/AMG mice lack a gap between the MGs due to the excessive branching that is already evident at P26 (Fig. 9). The distribution of HA in the developing eyelids was also investigated. Histologic sections of P1 eyelids stained with biotinylated HABP revealed that the developing tarsal plate presents strong HA expression (Fig. 10). Thus, the MG develops into an HA-rich tissue. Thus, we speculate that during early stages of MG development, when the epithelial-mesenchymal interaction leads to the invagination of epithelial...
cells into the mesenchymal tissue, the HA-rich mesenchymal tissue could serve as an important cue for regulating the proliferation and inward growth of epithelial cells.

**In Vivo and In Vitro Cell Proliferation**

Due to the increase in the size of MGs in the knockout mice, we investigated the effect of HA on MG cell proliferation, both in vivo and in vitro. HA in vitro had a stimulatory effect on meibocyte proliferation. Culturing hMGCs in the presence of both HMWHA and LMWHA led to a significant increase in cell proliferation, whereas culturing them in the presence of 4-MU, a chemical inhibitor that specifically blocks the synthesis of HA, led to a significant decrease in cell proliferation and at higher concentrations abrogated cell proliferation (Fig. 11). To assess the effect of HA on MG cell proliferation in vivo, HAS1−/−;HAS3−/−, and HAS2ΔΔMG mice were treated with EdU for 6 hours and eyelids were collected and processed for histology and whole-mount immunostaining. The distribution of EdU+ cells and HA were analyzed in sagittal sections of the eyelids. An increase in EdU+ cells and HA was observed in the HAS1−/−;HAS3−/− and HAS2ΔΔMG mice when compared with control mice (Fig. 12). As described earlier, the basal cells, which are the outer layer of cells that circumvent the MG, are surrounded by an HA-rich matrix. It has been previously shown, as with other holocrine glands, that the acinus of MGs is composed of an outer layer of basal cells that proliferate to generate new meibocytes that gradually move toward the center of the acinus. As the meibocytes move centripetally, they produce and store lipids. As the meibocytes fully mature

**FIGURE 8.** Whole eyelids were obtained from wt, HAS1−/−;HAS3−/−, and HAS2ΔΔMG mice at PD5, fixed, trimmed, stained with phalloidin (red), and mounted to enable flat mount. Images were captured under a confocal microscope. A white dashed line has been used to outline the developing MG. Nuclei were counterstained with DAPI (blue). Scale bar: 20 μm.

**FIGURE 9.** Whole eyelids were obtained from wt, HAS1−/−;HAS3−/−, and HAS2ΔΔMG mice at PD12 (A) and PD26 (B), fixed, trimmed, stained with phalloidin (red), and mounted to enable flat mount. The upper and lower eyelids were scanned under a confocal microscope. A white dashed line has been used to outline the developing MG. Scale bar represents 500 μm.
and reach the center of the acinus, they disintegrate and release portions of their cytoplasm containing the meibum into glandular ductules. Interestingly, the EdU+ cells within the MG are present within the HA-rich matrix (Fig. 12). Thus, together, our in vitro and in vivo data suggest that the HA matrix that surrounds the MGs could serve to support the highly proliferative cells. Moreover, as the meibocytes move centripetally, the loss of the HA matrix could trigger the maturation of these cells.

Differentiation Assay

To verify whether HA could play a role in regulating the differentiation of MG progenitor cells and lipid production, we used the hMGC differentiation assay. Lipid droplets accumulated in the cytoplasm of hMGCs cultured in differentiation media; however, the addition of HMWHA, LMWHA, ULMWHA, or 4-MU to the differentiation media inhibited lipid production (Figs. 13A, 13B). Seeding hMGCs on culture dishes coated with HMWHA or LMWHA also inhibited lipid production in comparison with control cells (Figs. 13C, 13D).

Hanging Drop Assay

To investigate whether HA surrounding the MGs plays a role in MG formation and differentiation, we used the hanging drop assay for the generation of a 3D culture system (Fig. 14). The hanging drop method involves culturing cells in a drop until they aggregate to form spheroids, after which they may proceed to form tissue-like cell aggregates and structures.52 After 4 days in serum-free culture conditions, MG epithelial cells formed nondenuse clusters (Fig. 14A). Interestingly, when the MG epithelial cells were cultured in the presence of HMWHA, they formed tissue-like structures with the formation of small spheroids emerging from the tube-like structures (Figs. 14A, 14B). In the presence of LMWHA, MG epithelial cells also organized into tissue-like structures, with small spheroids emerging from the tube-like structures, although the network was not as dense as that observed with HMWHA (Fig. 14A). When MG epithelial cells were cultured in the presence of ULMWHA, they formed nondenuse clusters similar to those observed with control untreated cells. The MG cells were then cultured for a further 6 days in the presence of FBS to trigger differentiation, culminating in a total of 10 days in vitro. When cultured in the presence of FBS, 50% of the untreated-control drops containing MG epithelial cells formed a nondenuse cluster of cells, while the other 50% formed a spheroid (shown in Fig. 14C). In the presence of serum and HMWHA, 100% of the drops containing MG epithelial cells formed a spheroid. In the presence of LMWHA and ULMWHA, the MG epithelial cells formed significantly smaller spheroids (Fig. 14C). After 10 days in vitro, the largest spheroid in each drop was measured, fixed, and processed for immunostaining. HMWHA induced the formation of significantly larger spheroids, when compared with the untreated control, LMWHA, and ULMWHA (Fig. 14D). In general, when using the hanging drop assay, factors that promote the formation of spheroids can be considered positive regulators of the developmental process.53,54 Thus, HMWHA could be considered to be a positive cue for development of the MG. Interestingly, phalloidin staining revealed that in the center of the spheroids, the MG epithelial cells started to form acini (Fig. 14E). A single acinus was seen in the center of control spheres, whereas multiple acini were observed within the HMWHA treated spheres, thus further confirming that HA promotes development of the MG (Fig. 14E). The ULMWHA-treated spheres, even though

### FIGURE 10.

HA (red) and K14 (green) staining in sagittal sections of eyelids from wt, HAS1−/−;HAS3−/−, and HAS2−/−/− mice at PD1. Staining revealed that HA is present throughout the mesenchymal component of the eyelid, and that HA expression is strongest at the tip of the eyelid. K14 staining reveals the developing MG growing into the HA-rich eyelid. Nuclei were counterstained with DAPI (blue). Scale bar: 50 μm.

### FIGURE 11.

The effect of HA on the proliferation of hMGCs was assayed in vitro by measuring BrdU incorporation. The hMGCs were seeded in the presence of 0.01% HMWHA; 0.01% LMWHA; 0.25 mM, 0.5 mM, 1 mM, or 2 mM 4-MU; or PBS as a vehicle control. After 24 hours, while the cells were in their exponential growth phase, BrdU was added to each well and left for 2 hours. Both HMWHA and LMWHA significantly promoted the proliferation of hMGCs, while 4-MU significantly inhibited proliferation at all concentrations used. *P ≤ 0.05.
smaller than the control spheres, also presented multiple acini (Fig. 14E). Interestingly, as seen in vivo, HA+ meibocytes were present in the outer layers of control spheroids, circumventing the developing acinus (Fig. 14F). Thus, a subset of meibocytes differentiate into HA-expressing cells and are present in the outer layers of the spheroids (Fig. 14F).

**DISCUSSION**

This study investigated the role of HA in eyelid and MG development and function. Our findings show, for the first time, that an HA-rich network surrounds the basal layer of MG acini and that the tarsal plate is an HA-rich tissue. Modifying HA...
levels within the eyelid leads to an increase in size and
morphologic alterations of the MG, demonstrating that HA
regulates MG morphogenesis.

HA is a ubiquitous component of cells and tissues and has
been extensively shown to play a vital role in development,
inflammation, and pathology. HA is a polysaccharide
that interacts with a wide variety of bioactive ligands, such as
growth factors, morphogens and their receptors, ECM
proteins, proteases, cytokines, chemokines, and enzyme
inhibitors. The specificity of the interactions between
HA and the plethora of ligands is dictated largely by the length
of the HA chain. The two most widely distributed forms of HA
are HMWHA and LMWHA, both of which are synthesized by
HASs. The biosynthesis of HA differs from that of other
members of the glycosaminoglycan family, which are synthe-
sized upon a protein core in the Golgi complex. In contrast,
HA biosynthesis takes place in the cytoplasm. The HAS
enzymes are located at the plasma membrane and the growing
HA is gradually released into the ECM during chain elongation.
Basic understanding of how the HAS enzymes regulate the
length of the growing HA chain during the biosynthetic
process, which drastically affects its physiologic function,
remains unknown. As the HA chain is synthesized, it is
incorporated into the ECM, and some groups have hypothe-
sized that the stiffness of the tissue during pathogenesis could
lead to a greater “pulling force” on the growing HA chain,
which would lead to the production of shorter HA chains. It
has also been speculated that HAS1 and HAS2 produce
primarily HMWHA, whereas HAS3 produces primarily
LMWHA; however, some groups have shown that all HAS
isoforms have the ability make both HMWHA and LMWHA. Interestingly, the Has knockout mice used in this study did not
display a loss of HA in the eyelids. Therefore, the Has knockout mice used in this study did not
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![Figure 14](https://arvojournals.org/)

**Figure 14.** The 3D hanging drop culture system was used to determine the role of HA in MG development. Ten-microliter drops of hMGCs were placed on the inside of a petri dish lid in the presence or absence of 0.05% HMWHA, 0.05% LMWHA, or 0.01% ULMWHA. The cells were maintained in culture for 4 days under serum-free conditions and images were collected (A). Thereafter, FBS was added at a final concentration of 10% to trigger
differentiation and images captured after 6 days after a total of 10 days in vitro (div) (B, C). The size of the largest sphere in each drop was measured
at 10 div, and HMWHA treated spheres were significantly larger in size (D). The spheres were fixed after 10 div and stained with phalloidin (white) and HA (red) (E, F). In control spheres, the development of an acinus could be noted in the center of the sphere, whereas in HMWHA- and ULMWHA-treated spheres, the formation of multiple acini was seen within the sphere (E). Cells in the outer layers of the spheres differentiated into
HA-producing cells (F). Nuclei were counterstained with DAPI (blue). Scale bar: 50 μm. *P < 0.05.
can, and tenascin are all hyaladherins and are generally found in tissues bound to HA-forming macromolecules that stabilize various matrices, such as in the bone and cartilage.66-68 These authors did not, however, investigate the staining pattern of HA in their study. Our work shows for the first time that HA is strongly expressed in the tarsal plate and that an HA matrix surrounds the basal cell layer of the MGs. We also show that HA is highly expressed within the mesenchymal component of the eyelid during eyelid development. HAS1 and HAS2\(^{1/2}\)/\(\Delta\)MG mice display precocious Meibomian budding during development and the adult mice present enlarged and elongated MG with an increased volume, primarily due to an increase in the number and size of acini. Excessive budding was noted in HAS1\(^{-/-}\)/HAS3\(^{+/-}\) and HAS2\(^{1/2}/\Delta\)MG mice by PD26, to the extent that there was no longer conjunctival tissue between individual MGs. Thus, our data suggest that an HA-rich mesenchyme plays a role during MG development and remodeling as a key regulator in the acinar budding process. Exacerbated acinar budding in HAS1\(^{-/-}\)/HAS3\(^{+/-}\) and HAS2\(^{1/2}/\Delta\)MG mice leads to the formation of enlarged and dysmorphic glands. Our data also demonstrate that HA plays a vital role in MG homeostasis, supporting proliferative MG precursor cells.

MGs are holocrine glands, meaning that meibocytes synthesize and accumulate lipids until they eventually undergo degeneration and disintegration as they discharge the meibum. Thus, the MGs must constantly replenish acinar cells that are destroyed as part of the secretory process. This requires that MG precursor cells in the basal layer proliferate and move centripetally, thereby providing a constant supply of newly maturing MG cells. Our findings show that the highly proliferative MG precursor cells are located within an HA-rich ECM. Together, our in vitro and in vivo data suggest that the HA matrix supports the MG precursor cells in the proliferative state. The hanging drop assay has been developed as a 3D in vitro cell culture model that more closely mimics in vivo histology. Thus, whether patients that are treated with eye drops containing HA as an active component develop epithelial hyperplasia should be explored in a clinical setting.

Over the past decade, there has been an increased pharmaceutical interest in HA, and many cosmetic products and Food and Drug Administration-approved medications, such as eye drops, currently contain HA as an active component. In recent years, the use of HA in ophthalmic products has grown exponentially, and of particular interest is the growing trend to add HA to eye drops for treating dry eye. Therefore, it is of vital importance to characterize both the expression profile and function of HA on the ocular surface before we can fully appreciate the potential impact of the long-term use of HA-based eye drops. This work is the first to show that HA is constitutively expressed in the basal layer of the MG and supports proliferating MG precursor cells. Moreover, changes in HA levels within the tarsal plate lead to the formation of dysmorphic enlarged MGs. Importantly, changes in HA levels...
lead to epidermal hyperplasia and the formation of epidermal outgrowths along the palpebral conjunctiva. Thus, our work suggests that clinicians should monitor patients using HA-based eye drops for epidermal hyperplasia and outgrowths in the palpebral conjunctival region that could lead to the loss of a smooth wiping surface. Moreover, our work lays the foundation for other studies to establish the role the eyelid ECM may play in ocular disorders for which the underlying pathogenesis remains elusive, such as the floppy eyelid syndrome.

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