Biomarkers for Progenitor and Differentiated Epithelial Cells in the Human Meibomian Gland

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ABSTRACT

The meibomian gland (MG) is a sebaceous gland that secretes through a holocrine process. Because such secretion requires the destruction of MG acinar epithelial cells, they need constant renewal and differentiation. The processes that promote these regenerative events in the human MG are unknown, nor is it known how to distinguish MG progenitor and differentiated cells. We discovered that Lrig1 and DNase2 serve as biomarkers for human MG progenitor and differentiated cells, respectively. Lrig1 is expressed in MG basal epithelial cells in the acinar periphery, a location where progenitor cells originate in sebaceous glands. DNase2 is expressed in the differentiated epithelial cells of the MG central acinus. Furthermore, proliferation stimulates, and differentiation suppresses, Lrig1 expression in human MG epithelial cells. The opposite is true for DNase2 expression. Our biomarker identification may have significant value in clinical efforts to restore MG function and to regenerate MGs after disease-induced dropout. STEM CELLS TRANSLATIONAL MEDICINE 2018;7:887–892

SIGNIFICANCE STATEMENT

Meibomian glands (MGs) are eyelid sebaceous glands that secrete through a holocrine mechanism. Because such secretion requires the destruction of MG acinar epithelial cells, they need constant renewal and differentiation. The processes that promote these regenerative events in the human MG are unknown, nor is it known how to distinguish MG progenitor and differentiated cells. The authors have discovered biomarkers for human MG progenitor and differentiated cells and have also learned that expression of these biomarkers is plastic and can be induced or suppressed repeatedly by changing the proliferation and differentiation status of these epithelial cells. These biomarker findings may have significant value in clinical efforts to restore MG function and to regenerate MGs after disease-induced dropout.

INTRODUCTION

Meibomian glands (MGs) are large sebaceous glands (SGs) located in the eyelids. The MGs are composed primarily of three types of epithelial cells: progenitor, differentiated, and ductal cells [1]. Progenitor epithelial cells (i.e., meibocytes) in these glands, as in skin SGs, appear to emerge from the acinar periphery and differentiate as they migrate across the acinus to the lateral duct [1]. Following terminal differentiation, meibocytes release their lipid-laden contents by holocrine secretion into the ductal system [1]. These secretions (i.e., meibum) spread onto the ocular surface from the orifices of the central duct at both the upper and lower eyelid margins and form the outermost lipid layer of the tear film. Meibum provides stabilization to, and prevents evaporation of, the tear film, which is critically important in maintaining ocular surface health [1–3].

Because holocrine secretion requires continual meibocyte destruction, these epithelial cells need continuous renewal and differentiation [1]. However, the processes that promote these regenerative cellular events in the human MG are unknown. Indeed, it is not even known how to distinguish progenitor and differentiated cells in this tissue. Such distinguishing ability, as might be realized with cellular biomarkers, would have tremendous value in clinical efforts to restore MG function and to regenerate these glands after disease-induced dropout (e.g., through tissue engineering, gene transfer, or cell therapy). The reason is that MG dysfunction (MGD), which is often paralleled by glandular dropout, affects hundreds of millions of people, is one of the most frequent causes for patient visits to eye care practitioners, and is the primary cause of evaporative dry eye disease [1, 2, 4–6]. There is no cure for MGD.
We hypothesize that leucine-rich repeats and immunoglobulin-like domains protein 1 (Lrig1) and DNase2 will serve as biomarkers for progenitor and differentiated cell populations in the human MG. Our rationale is that Lrig1 is a biomarker for proliferating progenitor cells in the pilosebaceous unit and that these precursor cells give rise to differentiated SG epithelial cells (sebocytes) [7–9]. Furthermore, lysosomal DNase2 is known to initiate the nuclear degeneration and holocrine secretion of sebocytes [10, 11].

The purpose of our study was to test our hypothesis by examining whether Lrig1 and DNase2 serve as biomarkers for progenitor and differentiated cells in the human MG. We also sought to determine whether these biomarkers are plastic and can be induced or suppressed by changing the proliferation and differentiation status of human MG epithelial cells. To help characterize the anatomical location of cells in the human MG, we used antibodies cytokeratins 6 (K6) and 14 (K14) to identify ductal [12, 13], as well as all [14, 15], epithelial cells, respectively.

**MATERIALS AND METHODS**

**Human Tissue**

Discarded and deidentified human eyelids were obtained within 12 hours after eyelid surgeries (two women, one man; age range, 70–82 years). The use of human tissues was approved by the Institutional Review Board of the Massachusetts Eye and Ear Infirmary and Schepens Eye Research Institute and adhered to the tenets of the Declaration of Helsinki. Tissue samples were immediately frozen in optimal cutting temperature compound (Tissue-Tek, Sakura USA, Torrance, CA) and processed for immunofluorescence and lipid staining.

**Cell Culture**

Immortalized human MG epithelial cells (IHMGECs) [16] were cultured in keratinocyte serum-free medium (KSFIM) containing 5 ng/mL epidermal growth factor (EGF) and 50 μg/mL bovine pituitary extract (BPE; Thermo Fisher Scientific, Grand Island, NY) to stimulate cell proliferation [17] (proliferation medium [PM]). After reaching 60%–70% confluence, cells were placed for varying intervals in one of the following media: KSFIM alone, PM, or serum-containing medium composed of 10% fetal bovine serum (Thermo Fisher Scientific) in equal volumes of Dulbecco’s modified Eagle’s medium and Ham’s F12 (differentiation medium [DM], Mediatech, Inc., a Corning Subsidiary, Manassas, VA) to induce cell differentiation [18]. To accelerate differentiation of IHMGECs, 10 μg/mL azithromycin (AZM; Santa Cruz Biotechnology, Dallas, TX) was added to the DM [19]. In some studies, culture media were switched multiple times between PM and DM. At experimental termination, cells were processed for immunofluorescent staining or immunoblotting.

**Immunofluorescence and Lipid Staining**

Human eyelid sections and IHMGECs were fixed with cold methanol for 15 minutes at −20°C. Following three phosphate-buffered saline (PBS) rinses for 5 minutes each, samples were blocked with 2% bovine serum albumin (BSA, Sigma-Aldrich Corp., St. Louis, MO) in PBS for 60 minutes, and then incubated overnight at 4°C in a moist chamber with antibodies specific for Lrig1 (ab214102, 1:100), cytokeratin 14 (ab181595, 1:500), cytokeratin 6 (ab18586, 1:500), DNase2 (ab8119, 1:100; Abcam, Cambridge, MA), or lysosomal-associated membrane protein 1 (LAMP-1; HA43, 1:15; Developmental Studies Hybridoma Bank, Iowa City, IA), or the BSA diluent. After additional PBS rinses, donkey anti-rabbit (ab150075, 1:500, Abcam) or donkey anti-mouse (2492098, 1:500, EMD Millipore, Temecula, CA) secondary antibodies were applied for 1 hour at room temperature. For neutral lipid staining, some eyelid sections were fixed in 4% paraformaldehyde for 15 minutes. Following additional washes, samples were exposed to LipidTOX Green neutral lipid stain (1:500, Thermo Fisher Scientific) in a humid chamber and for 30 minutes. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI, 1 μg/mL, Sigma-Aldrich) and samples were covered with VectaMount mounting medium (Vector Laboratories, Burlingame, CA), and observed with a confocal microscope (Leica TCS SP8, Leica Microsystems, Wetzlar, Germany).

**Immunoblotting**

Cells (n = 3 wells/experiment) were lysed in sodium dodecyl sulfate (SDS) Laemmli buffer (Bio-Rad Laboratories, Inc., Hercules, CA) supplemented with a 1% protease inhibitor cocktail and 5% β-mercaptoethanol (Sigma-Aldrich). Lysates were heated at 95°C for 15 minutes, separated by SDS-polyacrylamide gel electrophoresis on 4%–20% gradient gels (Thermo Fisher Scientific), and transferred to poly(vinylidene difluoride) membranes (Bio-Rad). Membranes were blocked with 5% milk or BSA in Tris-buffered saline containing 0.01% Tween-20 (TBS/T). Membranes were then incubated with primary antibodies to Lrig1 (ab36707, 1:500, Abcam), proliferating cell nuclear antigen (PCNA; D3H8P, 1:1000, Cell Signaling Technology, Danvers, MA), DNase2 (1:1000, Sigma-Aldrich), or β-actin (1:10000, Cell Signaling Technology) in TBS/T supplemented with 5% nonfat dry milk or BSA. Following an overnight incubation at 4°C, membranes were exposed to goat anti-rabbit or goat anti-mouse secondary antibodies coupled to horseradish peroxidase (1:5000, Sigma-Aldrich). Staining intensities were quantified with ImageJ software (https://imagej.nih.gov/ij/download.html).

**Statistical Analysis**

The significance of the differences between groups was determined by using Student’s unpaired, two-tailed t test (Prism 5, GraphPad Software, Inc., La Jolla, CA). Values with p < .05 were considered statistically significant.

**RESULTS**

**Lrig1 identification in the human MG**

To determine whether Lrig1 serves as a biomarker for progenitor cells in the human MG, we processed human tissues for the identification of ductal and acinar epithelial cell regions and then stained samples with antibodies to Lrig1. As shown in Figure 1A, K6 expression is limited to MG ductal cells, whereas K14 staining highlights both ductal and acinar epithelial cells. Immunofluorescence analysis of Lrig1 with or without K14 costaining demonstrated that Lrig1-positive cells are located solely in the acinar basal layer of the human MG (Fig. 1B). This presence of Lrig1 in the acinar periphery, and its absence in the central acinus, is analogous to that of progenitor cell distribution in other SGs.
Lrig1 expression in proliferating versus differentiating HMGECs

To examine whether Lrig1 expression is unique to proliferating, as compared with differentiating, cells, we cultured HMGECs in PM, DM, or DM + AZM media and processed them for morphological, immunofluorescence, and Western blot analyses. Proliferating HMGECs feature a distinctive, cobblestone morphology (Fig. 2A), which is associated with a pronounced expression of Lrig1 (Fig. 2B). In contrast, culture of HMGECs under differentiating conditions promotes a morphological shift to a large and flattened cellular appearance (Fig. 2A) and a loss of Lrig1 expression (Fig. 2B). The significant decrease in Lrig1 protein expression, which is induced by switching HMGECs from proliferating to differentiating media, was confirmed by Western blotting (Fig. 2C).

To verify the linkage between Lrig1 expression and cellular proliferation, we grew HMGECs until 70% confluency, and then cultured them in either basal KSFM or PM media for an additional 3 days. After this period, we analyzed cell lysates for both Lrig1 and PCNA, which is an indicator of cell cycle entry [20]. As shown in Figure 3, the expression of both Lrig1 and PCNA proteins were significantly enhanced in PM conditions.

DNase2 expression in the human MG and in HMGECs

To determine whether DNase2 serves as a protein biomarker for differentiated cells in the human MG, we processed human tissues for the identification of neutral lipids and lysosomes in differentiated cells and then stained samples with antibodies to DNase2. As anticipated, the maturing, lipid-containing cells were situated in the central region, but not the basal layer, of the MG acinus (Fig. 4A, 4D). DNase2 expression was identified in, and restricted to, the lipid-laden lysosomes in these differentiated cells (Fig. 4B, 4C, 4D). DNase2 could not be detected within the progenitor cell area of the acinar periphery (Fig. 4B).

Figure 1. Identification of K14, K6, and Lrig1 in the human meibomian gland (MG). (A): K14 is present in both ductal and acinar epithelial cells, whereas K6 expression is restricted to ductal cells. (B): Lrig1-positive cells are located only in the basal layer of the MG acinus. Scale bar = 50 μM. Abbreviations: K6, cytokeratins 6; K14, cytokeratins 14.

Figure 2. Differential appearance of Lrig1 in proliferating versus differentiating human meibomian gland epithelial cells (HMGECs). In parallel with a change in cellular morphology (A), Lrig1 expression in proliferating HMGECs (B) (PM frame) appears to be lost when cells differentiate (B; DM and AZM frames). (C): This decrease in Lrig1 levels was confirmed by Western blot (n = 3 experiments, **p < .01). Scale bar = 50 μM. Abbreviations: AZM, azithromycin; DM, differentiation medium; PM, proliferation medium.
Plasticity of Lrig1 and DNase2 biomarkers in HMGECs

To determine whether the Lrig1 and DNase2 biomarkers are plastic and can be induced or suppressed by changing the proliferation and differentiation status of HMGECs, we performed two types of reversibility studies. First, we cultured HMGECs in PM for 3 days, switched the media to DM for another 3 days, and then returned the cells to PM (Fig. 6A). Second, we altered the media sequence, and began by placing the HMGECs in DM media, then switching to PM, followed by DM (Fig. 6C). As shown in Figures 6B and 6D, we discovered that proliferation stimulates, and differentiation suppresses, Lrig1 expression in HMGECs. The opposite is true for DNase2 expression (Fig. 6B, 6D).

DISCUSSION

Our results demonstrate Lrig1 is expressed in the basal epithelial layer of the human MG, and not in the differentiated cells.

Figure 3. Stimulation of Lrig1 expression in immortalized human meibomian gland epithelial cells. (A) Cells were cultured in basal KSFM or PM media. The latter promoted both Lrig1 and PCNA appearance, as shown by Western blot (n = 3 experiments, *p < .05) (B). Abbreviations: KSFM, keratinocyte serum-free medium; PCNA, proliferating cell nuclear antigen; PM, proliferation medium.

Figure 4. Cellular location of DNase2 in the human meibomian gland (MG). To identify neutral lipids and lysosomes, cells were stained with LipidTOX [19] and antibodies to LAMP-1 [31]. LipidTOX staining of neutral lipids is present in the epithelial cells of the acinus, but not the basal layer (A). DNase2 staining localizes in central cells of the acinus (B). DNase2 is located in neutral-lipid (D) containing lysosomes in the human MG. Note the cytoplasmic lipid droplets (D), which are also found in sebocytes [32]. Scale bar = 50 μM. Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; LAMP-1, lysosomal-associated membrane protein 1.

Figure 5. Differential expression of DNase2 in proliferating versus differentiated human meibomian gland epithelial cells (HMGECs). DNase2 is manifest in HMGECs when cultured in DM or AZM, but not in PM, as shown by immunofluorescence (A) and Western blot (B) (n = 3 experiments, **p < .01). Scale bar = 50 μM. Abbreviations: AZM, azithromycin; DM, differentiation medium; PM, proliferation medium.
in the central section of the MG acinus. In contrast, DNase2 is expressed in the differentiated epithelial cells of the MG central acinus, and not in peripheral basal cells. Our findings also show that proliferation stimulates, and differentiation suppresses, Lrig1 expression in HMGECs. The opposite is true for DNase2 expression. These results support our hypothesis that Lrig1 and DNase2 serve as biomarkers for progenitor and differentiated cells in the human MG.

This differential distribution of Lrig1 in the basal layer, and not in the central area, of the MG is analogous to that found in SGs [1, 7–9]. The SG basal layer is the source of Lrig1-positive, proliferating progenitor cells, which give rise to differentiated sebocytes in the central region [7–9, 21, 22]. Thus, it appears that meibocytes are generated from Lrig1-positive progenitor cells in the MG basal layer [1, 23]. This finding is consistent with the observation that MGs display a gradient of meibocyte maturation, with more undifferentiated, immature cells in the basal layer, and more mature, lipid-containing cells in the center of the acinus [21, 22].

Lrig1 has also been identified as a marker of human corneal [24], epidermal [8] and intestinal [25, 26] stem cells, and the majority of Lrig1-positive intestinal stem cells are proliferating cells [26]. Other studies, though, have reported that Lrig1-expressing stem cells are predominantly quiescent [8, 25], and that Lrig1 serves as a negative regulator of EGF receptor signaling to suppress stem cell proliferation [8, 27, 28]. How, then, do we explain our finding that EGF and BPE promote proliferation, and stimulate the accumulation of Lrig1, in HMGECs? It is possible that Lrig1 may act within a negative feedback loop. As demonstrated by Gur et al. [27], Lrig1 expression increases in response to growth factor-induced proliferation [17], in order to attenuate cell proliferation and maintain epithelial quiescence.

We discovered that DNase2 is expressed only in the lipid-containing differentiated cells of the human MG. This cellular location likely reflects DNase2’s ultimate role, as in sebocytes, to initiate nuclear degeneration and holocrine secretion [10, 11]. This autolytic process appears to be driven by endolysosomal DNase2, and results in a unique form of programmed cell death [10, 11]. Of interest, we have observed that androgen treatment of HMGECs upregulates the gene expression for DNase2 (unpublished data). This response is associated with an androgen-induced increase in genes promoting cellular differentiation and a decrease in those driving cell proliferation [29]. The nature of this hormone response seems to mimic that of AZM, which decreases cell proliferation [19], stimulates differentiation, and enhances DNase2 accumulation in HMGECs.

We found that expression of the Lrig1 and DNase2 biomarkers is plastic and that both biomarkers can be induced or suppressed repeatedly by changing the proliferation and differentiation status of HMGECs. Such reversibility has also been observed in primary human corneal cells [30], indicating that the immortalized HMGECs share characteristics of primary cells. Indeed, we have previously found that many of the genetic responses of immortalized HMGECs are the same as those of primary HMGECs [18]. These findings suggest that this MG cell line, which features a normal karyotype, represents an appropriate model in vitro to study HMGEC dynamics [16]. Furthermore, it may be that primary HMGECs have similar biomarker plasticity, which could have implications for MG regeneration in vivo.

**CONCLUSION**

In summary, our results support our hypothesis that Lrig1 and DNase2 serve as biomarkers for progenitor and differentiated cells, respectively, in the human MG. Future studies will help determine the precise role of these proteins in HMGECs.

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AUTHOR CONTRIBUTIONS

D.A.S. and Y.L.: conception and design; H.-T.X., D.C., M.P.H. and Y.L.: data collection; H.-T.X., D.A.S. and Y.L.: data analysis and interpretation; M.P.H.: provision of study material; H.-T.X., D.A.S., W.R.K. and Y.L.: manuscript writing; H.-T.X., D.A.S., D.C., M.P.H., W.R.K. and Y.L.: final approval of manuscript; D.A.S.: funding acquisition.

DISCLOSURE OF POTENTIAL CONFLICT OF INTEREST

D.A.S. discloses patent holding, research funding and ownership interest with Singularis/Lybris Biopharma - Lubricin product in development and has no relationship to stem cells. All other authors indicated no potential conflicts of interest.

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