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Meibomian gland stem/progenitor cells: The hunt for gland renewal

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Abstract

Meibomian glands (MGs) secrete lipid (meibum) onto the ocular surface to form the outermost layer of the tear film. Proper meibum secretion is essential for stabilizing the tear film, reducing aqueous tear evaporation, and maintaining the homeostasis of the ocular surface. Atrophy of MG as occurs with aging, leads to reduction of meibum secretion, loss of ocular surface homeostasis and evaporative dry eye disease (EDED). Since MGs are holocrine glands, secretion of meibum requires continuous self-renewal of lipid-secreting acinar meibocytes by stem/progenitor cells, whose proliferative potential is dramatically reduced with age leading to MG atrophy and an age-related meibomian gland dysfunction (ARMGD). Understanding the cellular and molecular mechanisms regulating meibocyte stem/progenitor cell maintenance and renewal may provide novel approaches to regenerating MG and treating EDED. Towards that end, recent label retaining cell and lineage-tracing experiments as well as knock-out transgenic mouse studies have begun to identify the location and identities of meibocyte progenitor cells and potential growth and transcription factors that may regulate meibocyte renewal. In addition, recent reports have shown that ARMGD may be reversed by novel therapeutics in mice. Herein, we discuss our current understanding of meibocyte stem/progenitor cells and the hunt for gland renewal.

Keywords

Meibomian gland; Homeostasis; Stem cells; Evaporative dry eye disease; Age-related meibomian gland dysfunction; Atrophy

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Declaration of competing interest

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1. Introduction

The tear film covering the cornea and ocular surface is comprised of an inner aqueous/mucous layer and an outer lipid layer that are vital to ocular surface homeostasis [1]. Mucins in the tear film are mainly synthesized by conjunctival goblet cells and the corneal epithelium, and the bulk of the aqueous tears is secreted by the lacrimal glands. The lipid layer is produced by the tarsal, meibomian glands (MGs) of the inner eyelid that synthesize meibum lipids that are secreted onto the eyelid margin and spread over the ocular surface with each eyelid blink. Extensive studies have established that an important and critical function of meibum is to lower the surface tension of the tear film to reduce aqueous tear evaporation [2,3]. Furthermore, alterations in the quantity and/or quality of meibum is known to lead to tear film instability [4–7].

Meibomian gland dysfunction (MGD) is the leading cause of evaporative dry eye disease (EDED), and MGD associated dry eye is recognized as the most prevalent disorder of the ocular surface. Patients with MGD suffer from recurrent irritations or foreign body sensations in their eyes, which can greatly affect their quality of life and mental health. With the wide availability of infrared meibography [8], MG atrophy, glandular dropout and distortion have been recognized as the most frequent structural abnormalities in MGD [9,10]. Of note, MG atrophy or dropout progress with aging, accompanied by deteriorating MG function. Severe MG atrophy leads to MG hyposecretion and poor delivery of meibum to the tear film. However, little is known about the MG homeostasis and regeneration. Current treatments for MGD are mostly palliative, aiming to relieve the dry eye symptoms or putative glandular occlusion. To date, there is no specific treatment that is known to restore the function or structure of MG, and it remains unknown whether atrophic age-related MGD (ARMGD) can be reversed.

2. Physiology of meibomian glands

2.1. Anatomy and secretion of meibomian glands

Meibomian glands are embedded in a vertical row within the tarsus, a dense connective tissue matrix located underlying the orbicularis oculi muscle of the upper and lower eyelids. In the human, there are approximately 20–25MGs in the lower and upper eyelids, respectively [11]. Individually, MGs are composed of a long central duct with branching ductules that connect to secretory acini (Fig. 1) [12]. At the distal end, the central duct opens onto the eyelid margin at the orifice, located anterior to the mucocutaneous junction, where dry skin meets wet conjunctiva, and as such, the central duct is anteriorly lined by keratinized, stratified squamous epithelium that proximally becomes non-keratinized to line the majority of the duct and ductules [13]. Mouse MGs are similar in many ways to human MGs, and contain a central duct with budding acini underlying the orbicularis oculi muscle and adjacent to the conjunctiva (Fig. 2). Nevertheless, there are structural differences between human and mouse in the basic anatomic connections between the ductules and acini (Fig. 3). While human ductules are short and independently extend from the long central duct to connect to single acini (Fig. 3A), in the mouse there is a complex array of highly branched ductules connecting to small acini near the duct orifice and much larger acini at

the proximal terminus of the gland (Fig. 3B). Other than these anatomic differences, the mouse and human MGs are thought to be very similar regarding the synthesis of meibum and the effects of age [14–16], albeit differences in cellular dynamics, lineage commitment and plasticity during MG homeostasis and regeneration have yet to be explored.

3. Cellular dynamics of the meibomian glands

Meibomian glands secrete meibum using a holocrine mechanism of secretion that involves the release of secretory products by the whole cell through a process of cellular disintegration; thus far an unexplored programmed cell death or 'meiboptosis', a mechanism similar to that used by sebocytes. Different from merocrine glands, i.e., lacrimal and salivary glands that secrete products through exocytosis during the extended lifetime of the cell, secretion by holocrine glands requires the continual renewal of meibocytes by an undifferentiated meibocyte progenitor cell population. As first shown by Olami et al. in the rat meibomian gland, putative meibocyte progenitor cells located in the basal compartment surrounding the acini undergo continuous cell division every 4.1 days as measured by ^{3}H-thymidine uptake [17]. Given that >25% the acinar basal population is in cycle at any one time as measured by Ki67 labeling in both young human and mouse meibomian glands [15,16], the entire meibocyte progenitor population likely turns over every 4 days. Progenitors meibocytes then leave the basal compartment and migrate centripetally toward the center of the acinus, while undergoing a differentiation processes that involves, in part, the expression of lipid synthesizing genes, active lipid synthesis and accumulation of meibum lipids [18]. While several stages of differentiation have been described based on the histologic and ultrastructural appearance, meibocytes ultimately reach the center of the acinus where they begin to disintegrate and release their secretory products into the meibomian gland ductal system. As measured by Olami et al., this differentiation process takes approximately 9 days in the rat [17].

Unlike merocrine glands where secretion is assisted by the contractile activity of myoepithelial cells surrounding the secretory acini, secretion of meibum is thought to be passive and dependent on mechanical forces generated by the overlying orbicularis oculi muscle during eyelid blinking [19]. Contraction of the orbicularis oculi is known to exert a mechanical force or pressure on the eye that can vary between 10.3 mmHg with a normal blink and up to 51 mmHg with a forced blink [20]. Since the meibomian gland lies between the muscle and the eye, this force is also exerted on the meibomian gland duct and is thought to cause the secretion of meibum out onto the lid margin. By contrast, Riolan's muscle, which surrounds the distal portion of the central duct near the orifice, contracts during relaxation of the orbicularis muscle and is thought to inhibit secretion during eyelid opening [19], though more recent studies showing continued meibum secretion during eyelid opening leaves this mechanisms open to question [21].

While the effects of aging on the cellular dynamics of the meibomian gland are poorly understood, past studies have shown that there is a significant age-related loss in the proliferative potential of meibocyte progenitor cells in both humans and mice. Using Ki67 immunocytochemical staining to identify cycling cells, the number of cycling cells decreases below 10% of basal cells by 60 years of age in humans and 1 year of age in mice [15,16].

This decrease in proliferation is accompanied by a decrease in the immunocytochemical staining of the nuclear receptor, peroxisome proliferator activated receptor gamma (PPARγ). While PPARγ activation is required for differentiation and expression of enzymes related to lipid synthesis in sebaceous glands and adipocytes [22], western blotting of proteins extracted from the mouse tarsal plate and meibomian glands show a complete loss of cytoplasmic and a 50% decrease in the nuclear expression of PPARγ that occurs with age [23]. These molecular changes also coincide with a decrease in the size of the meibomian glands in mice suggesting that aging also causes MG atrophy in the mouse, similar to that in the human [24,25].

3.1. Meibocyte progenitor cell population

Epithelial cells are known to express an array of type I (acidic) and type II (basic/neutral) keratin proteins that dimerized and then polymerize to form intracellular keratin filaments, whose expression pattern is unique for different stages of differentiation and tissue type [26]. In the skin, differentiated cells characteristically express the keratin pair, Krt1/10, that is associated with keratinization or cornification that is important to the formation of a water barrier. These differentiated cells are derived from progenitor cells that are located in the basal compartment and express Krt5/14, whose expression is lost as the cells migrate into the suprabasal layer and begin to express Krt1/10 keratins. Basal cells expressing Krt5/14 are highly proliferative and are derived from putative skin stem cells located in the interfollicular epidermis or the hair follicle and are sometimes referred to as transient amplifying cells (TACs) that are involved in renewal and repair processes [27,28].

Similarly, TACs are present in the meibomian glands as originally shown by Olami et al. [17], and more recently by Parfitt et al. [25] In the later report, Krt5 expression was identified in proliferating basal cells of both the duct and acinus. In the duct, basal cells appeared to either express Krt5 alone, or co-express Krt5 with Krt6a, another acidic keratin that is characteristic of differentiated conjunctival epithelium. Upon exit from the basal layer, ductal epithelial cells lose Krt5 expression and then express Krt6a in the proximal portion of the duct, or Krt1/10 in the very distal/anterior portion of the duct. Whether these differentiated ductal epithelial cells are derived from the same or different stem cell population remains unclear.

By contrast, basal Krt5 positive cells in the acinus are Krt6 negative, and they lose Krt5 expression with differentiation to acinar meibocytes. As progenitor meibocytes start to differentiate they begin to express PPARγ, the transcription factor that controls the expression of genes specific for lipid synthesis and meibogenesis [18]. As shown in Fig. 4, meibomian glands in young mice (2 month old) show a very high proportion of cells that stain for PPARγ, with many of these cells showing expression of Krt5, the positive basal meibocyte progenitor cell marker (Fig. 4 A, arrows). Interestingly, the basal meibocyte progenitor cells show a gradation of PPARγ staining with the more intensely stained cells appearing to begin migration up to the suprabasal, differentiating compartment as they lose Krt5 expression. Since previous in vitro experiments have shown that PPARγ expression and activation initiates both cell cycle exit and expression of genes involved in meibum synthesis [29], this data suggests that PPARγ is a key initiator of the transition from

meibocyte progenitor cell to meibocytes and that this process is initiated in the basal cell compartment of acinus. Furthermore, the total number of PPARγ positive cells as well as the percentage of PPARγ labeled progenitor cells is significantly reduced in old mouse meibomian glands, going from 39 ± 6% total and 35 ± 7% basal in young to 20 ± 3% total and 21 ± 5% basal in old mice ($p < 0.01$ and < 0.05, respectively). This finding suggests that not only is the proliferative rate of renewal of meibocytes decreased during aging, but also the rate of meibocyte differentiation; a possibility that would further impact the synthesis and delivery of meibum to the ocular surface. Certainly, the effects of age on the rate of meibocyte renewal and differentiation need deeper study.

3.2. The search for the putative meibocyte stem cell

To begin to identify the meibomian gland stem cell population, prior studies have pulsed mice with ^{3}H-thymidine and bromodeoxyuridine (BrdU) to label cycling cells, and then chased mice for various times to identify label retaining cells (LRC) in the meibomian gland, the hallmark of slow-cycling stem cells. Studies by Olami et al. using adult rats injected with a single dose of ^{3}H-Thymidine to label fast cycling cells and then chased for up to 28 days, suggested that acinar progenitor cells were derived from putative MG stem cells located circumferentially at the opening of the acinus [17]. In another study by Lavker et al., injected BrdU into 3 day old, neonatal SENCAR mice and chased for 6–8 weeks [30]. Using this longer pulse-chase paradigm, MG LRCs appeared located more preferentially in the ductal epithelium, leading them to conclude that the ductal epithelium was enriched in MG stem cells and that renewal of MG cells was strikingly similar to that of the hair follicle. However, the findings by Lavker et al. remain unclear as the details have only been published in abstract form.

More recent studies using genetic labels have further explored this question by performing LRC and lineage tracing studies using the Krt5 and Krt14 promoters to drive GFP labeled H2B histone expression or the rainbow cassette to express multiple fluorescent markers [31,32]. In the LRC studies, bitransgenic mice expressing the Tet-off Krt5 promoter to drive H2B-GFP expression were labeled from birth to 2 months of age. Mice were then fed doxycycline to turn off H2B-GFP expression and chased from up to 58 days. LRCs were then localized and quantified using a 3D reconstruction method consisting of plastic tissue embedding, serial thin sectioning, immunostaining and 3D reconstruction [33]. In these studies two groups of Krt5^{+} LRCs were identified at the interface between the Krt6 positive ductular epithelium and the Krt6 negative acinus that were characterized by either co-expression of both Krt5/Krt6 that might give rise to progenitor ductal epithelial cells or expression of only Krt5 that might give rise to progenitor meibocytes (Fig. 5) [32]. Interestingly, there were very few LRC that were identified in this study, suggesting only 1 or 2 LRCs/acinus remaining after 58 days chase. Further characterization of the meibocyte LRCs showed that they were all Sox9 positive, Blimp1 negative and PPARγ negative. More recently these findings have been repeated using the K14/H2B-GFP mouse, which produced similar findings to that obtained with the Krt5/H2B-GFFP mouse (data not shown) and together suggest that the Krt5/14 positive progenitors cells located at the junctional regions between acini and ductules are critical for the MG homeostasis and renewal.

Lineage tracing studies have also been performed using the Confetti transgenic mouse that can stochastically express through DNA recombination one of four fluorescent probes when driven by the tamoxifen sensitive expression of CreER fusion recombinase driven by Krt14 promoter in stem/progenitor cells [34]. One year after tamoxifen injection, confetti mice showed isolated acini labeled by different fluorescent probes such that the entire acinus was labeled by a single probe, therefore suggesting that meibocytes within an individual acini are derived from a single stem cell and are completely self-renewing under normal homeostatic conditions [32]. Importantly, labeled acini showed no extension of label into adjacent Krt6a positive ductal epithelium, indicating that meibocyte stem cells do not give rise to both meibocytes and ductal epithelium. A similar pattern was also detected for hair follicle sebaceous glands. Regarding the ductal epithelial stem cells, isolated regions of duct showed labeling by the same probe suggesting that ductal epithelial renewal was achieved by isolated stem cells along the duct perhaps arising from the Krt5/Krt6a positive LRC at the interface between the duct and acini.

Taken together, these LRC/Lineage tracing studies strongly suggest that under normal homeostatic conditions there are two stem cell populations, one giving rise to the acinar meibocytes that are $Krt5^{+}/Krt6^{-}/PPAR\gamma^{-}/Sox9^{+}$ and one giving rise to the ductal epithelium that are $Krt5^{+}/Krt6^{+}/PPAR\gamma^{-}$ (Fig. 5, Hypothesis A). While this is the most consistent interpretation of the LRC/Lineage tracing data, one cannot rule out the possibility that either stem cell may potentially give rise to the other opposite cell lineage and are potentially bipotent stem cell populations (Fig. 5, Hypothesis B). Under the conditions of Hypothesis A, renewal of the meibocyte and/or ductal epithelial progenitors cells may be restricted by gland atrophy due to the loss or exhaustion of these stem cell populations under homeostatic conditions. Conversely, following injury and repair, the bipotent capability of the ductal and/or meibocyte stem cells may allow for regrowth of the meibomian glands as proposed by Hypothesis B. Such may be the case following conditional knockout of FGF receptor 2 that leads to the degeneration of acini, later followed by ductal atrophy [35]. Interestingly, when these conditional knockout mice were followed for 30–60 days, there was substantial regeneration of the formerly degenerated glands [36]. These findings would support either the presence of a single stem cell population, or the recruitment of a ductal stem cell to provide new meibocyte stem cells under regenerative or reparative conditions. The existence of either unipotent or bipotent progenitors giving rise to both the meibocyte and ductal epithelial progenitors awaits further investigation.

3.3. Proposed biomarkers for MG stem/progenitor cells

To date, biomarkers that can identify MG stem/progenitor cells remain elusive. Parfitt et al. reported that 100% of the $Krt5^{+}/Krt6^{-}$ LRCs expressed SOX9, and very few were detected that expressed Blimp1, the putative progenitor markers of SGs [31]. However, the expression of both Blimp1 or SOX9 was not restricted in basal cell population and therefore cannot serve as specific markers of MG stem/progenitor cells, though their respective function has yet to be clarified [37]. In addition, CD147, an inducer of extracellular matrix metalloproteinase, labels the basal meibocytes and ductal epithelial cells. Knockout of CD147 causes severe MG atrophy in mice, suggesting an important role of CD147 in maintaining MGs [38]. Moreover, Lrig1 is proposed as a putative marker

for distinguishing the basal and differentiated meibocytes [39]. Of note, a recent study exploiting lineage tracing reports that Krox-20, a zinc finger transcription factor, may mark MG stem cells populations during development and homeostasis [40,41]. Furthermore, the Krox-20$^+$ lineage cells are also capable of generating the entire MG, suggesting that the Krox-20-positive stem/progenitor cells give rise to not only ductal epithelial cells, but also acinar cells during morphogenesis [41]. Another interesting finding of this study was that the Krox-20$^+$ cells do not express Krt14 in the MG prior to P12, but Krt14-Cre effectively induced Krox-20 deletion. While this shift in expression to Krt14 in Krox-20$^+$ cells may be caused by leaky expression of Krt14-Cre, the finding may also suggest the presence of a complicated crosstalk mechanism that governs linage commitment and cell fate in MG during development. Nevertheless, Krox-20 may serve as a promising stem/progenitor marker for MGs, while its role in MG repair and regeneration awaits further study.

3.4. Comparison of putative stem/progenitor markers between SG and MG

MG and SG, both are holocrine glands, develop from primordial ectoderm, and share certain similarities regarding lineage commitment and stem cell properties (Fig. 6). Hence, initial exploration into MG stem/progenitor cell properties have drawn heavily on the earlier and more detailed studies in SGs. As such, Lrig1 is one of the well-characterized stem cell markers associated with SG homeostasis [42, 43]. Lrig1$^+$ keratinocytes located at the junctional zone of hair follicles contribute to the SG renewal (Fig. 6A) [43]. Similarly, Lrig1 marks the MG acinar basal layer [39,44], though its requirement for MG renewal needs further study. Blimp1 is expressed in the base of SG and has been proposed as a marker for SG progenitors [45]. However, Blimp1 was not localized to MG LRCs [31], while expression was found in post-mitotic and terminally differentiated SG and MG cells [46]. Hence, Blimp1 is no longer regarded as a proper marker for SG stem cells, nor should be considered a marker for MG stem cells. Lgr6$^+$ and Krt15$^+$ keratinocytes in the upper bulge/isthmus region of hair follicles can also generate sebocytes [47,48], but their expressions in MG have not been characterized. Furthermore, Gata6 is expressed in the SG ductal cells and plays important role in controlling the proliferation and differentiation of sebocytes (Fig. 6) [49]. Whether Gata6 can serve as a specific stem cell marker for MG ductal epithelial cells mandates further investigation.

Taken together, these studies suggest that there are perhaps multiple stem/progenitor cell populations within the meibomian gland that take part in MG development, homeostasis and repair (Table 1). Identifying the specific identities of these populations and the mechanisms controlling their function will require additional studies, perhaps using lineage tracing paradigms to fully identify and characterize their roles.

4. Pathophysiology of meibomian gland atrophy

4.1. MG atrophy secondary to obstructive MGD

MG atrophy and dropout are the pathognomonic features of MGD, and conventionally thought to be caused by obstruction of the meibomian gland secretory duct leading to cystic dilation of ducts and acini causing a disuse acinar atrophy and a hyposecretory disease [50]. Other histopathologic features that have also been identified in human MGD including;

basement membrane thickening, granulation, keratinization of the MG ducts and orifices and lipogranulomatous inflammation [51]. The first animal models showing dysfunction of the meibomian glands were associated with abnormal thickening and growth of the keratinized ductal epithelium in rabbits following long-term topical epinephrine treatment and polychlorinated biphenyl toxicity in primates [52–54]. Early mouse models also showed filling of the meibomian glands with keratinized ductal epithelium [55], suggesting that one mechanism underlying the development of MGD was hyperkeratinization of the gland. Today, the prevailing view is that hyperkeratinization of the duct leads to a narrowing of the of the MG orifice and gland obstruction and impaired excretion of meibum that gradually leads to cystic dilation of the acini and the duct with progressive stagnation and compressive glandular atrophy [13]. More recent MGD mouse models that directly impact the meibum lipid quality, either through changes in diet [56] or modifying expression of enzymes associated with meibum synthesis [57–59], provide potential alternative mechanisms that could underlie obstructive MGD. Taken together, these newer models indicate that normal lipid composition and quality may be more critical to MG homeostasis and may impact both the renewal and growth of the meibomian gland duct and meibocyte populations.

4.2. Age-related MG atrophy

Aging is a major risk factor for MGD and EDED, although the mechanism underlying atrophy is not specifically known, nor how it may or may not differ from atrophy caused by 'ductal obstruction'. The atrophic process of ARMGD in humans is readily detected as early as 25 years of age and continues to progress beyond 60 years of age [60–63], and manifests both morphological and functional changes with progressive glandular dropout and reduced meibum secretion [61,64]. In animal studies, the ARMGD exhibits compromised capability for self-renewal with possible reduced meibum secretions [16,65]. It is highly conceivable that senescence leads to altered stem cell functions and imbalanced cell differentiation. As mentioned earlier, studies have confirmed that the proliferation of meibocytes decreased remarkably in atrophic MGs [15,16], an observations also confirmed in cadaver tissue [66,67]. In addition, uniformed thinning and atrophy of the ductal epithelium and shrinkage of the acinar area were noted in the aged human MG. Nevertheless, thickening of the keratinized epithelium or hyperkeratinization is not a characteristic of the mouse model of ARMGD [25,68]. Furthermore, as discussed above, there is a marked loss in the expression and post translational modification of PPARγ, the fatty acid activated nuclear transcription factor, required and essential for lipogenesis and adipocyte/meibocyte/sebocyte differentiation. How these changes in this key transcription factor effect meibum quality and quantity of secretion is not known, but may contribute to the compositional changes that have been identified during aging that include a lower ratio of non-polar to polar lipids, that has also been associated with the clinical signs and symptoms dry eye [69–71]. Genetic ablation of nicotinamide adenine dinucleotide (NAD+)-dependent 3β-hydroxyl-steroid dehydrogenase (3β-HSD) has also recently been shown to both nullify local steroidogenesis and lead to meibomian gland atrophy. Interestingly, activation of 3β-HSD through boosting availability of its coenzyme, NAD+, improved glandular cell proliferation and increased gland size suggesting that NAD + levels and the action of 3β-HSD may play a role in MG atrophy during aging [72]. Nevertheless, the

underlying mechanisms associated with ARMGD remain incompletely understood, and in general the aging model has yet to be fully appreciated nor explored to its full extent.

5. Animal studies regarding MG regrowth and novel therapeutic strategies

As MG undergoes progressive atrophy with age, the search regarding whether the atrophic gland is capable of regrowth has become increasingly important. As a holocrine gland, MGs may possess robust renewal and recovery potentials after injury, perhaps similar to that of the SG. However, the recovery and regenerative capabilities of MGs remain poorly understood. Previous studies have observed spontaneous regrowth of atrophic MGs in the *Fgfr2*^{CKO} mice after ceasing conditional knock down [36]. This finding suggests that the surving murine MGs after induced atrophy still possess a level of repair and regeneration, which may be determined by the integrity of residual ductular structures that might provide new, or contain residual meibocyte stem/progenitor cells.

Recent studies have demonstrated that MG atrophy in the aged C57Bl/6 mouse could be ameliorated by novel therapies targeting the proliferative activity of the basal meibocytes [44,73]. Fan et al. found that treatment with a synthetic PEDF-derived 29-mer peptide effectively promotes the growth of atrophic MGs in aged mice, presumably via stimulating the proliferation of meibocyte progenitor cells and enhancing the number of ΔNp63 and Lrig1-positive cells [44]. Similarly, topical treatment of periplocin, a natural agonist of Na+/K + ATPase, has been reported to be effective in enhancing the proliferation and lipid synthesis of the MGs in aged mice via the SRC signaling pathway [73]. Together, these studies support the view that MG can be induced to undergo regeneration and potentially rescued or reverse gland atrophy by short-term pharmacological treatment to promote meibocyte renewal. However, cautions should be exercised when considering these specific targets as potential therapeutics for ARMGD, as continuous activation of basal meibocyte might accelerate the exhaustion of stem cells in MG. Long-term efficacy and safety of the above strategies mandates further scrutiny.

Similarly, treatment of MG atrophy secondary to obstructive MGD might also be reversed with interventions, though this possibility remains to be answered. A recent study reported that the possible obstructive MGD model induced by Stearoyl-CoA desaturase 1 (SCD1) deficiency could be rescued by inhibition of ceramide synthesis [74]. SCD1 plays a central role in regulating lipid synthesis in MGs, and depletion of SCD1 leads to decrease of lipid synthesis, increased synthesis of ceramides, atrophy of MG acini and increased duct keratinization. Of note, blocking of endogenous ceramide synthesis effectively ameliorated glandular atrophy and ductal keratinization induced by SCD1 inhibition. In addition, diquafosol, a purinergic P2Y2 receptor agonist, might stimulate lipid release and facilitate meibocytes maturation in vitro. Application of 3% diquafosol eye drop was reported to be favorable in improving MG structure in the Cu, Zn-superoxide dismutase 1 (Sod1) knockout mice [75]. In SG and MG, extensive evidence has confirmed that androgen could up-regulate the genes related to lipid synthesis and downregulate genes associated with the keratinization process [76–78]. For instance, topical treatment of DHEA eye drops increased the lipid secretion and minimized the ductal keratinization and acinar atrophy in rats [79].

Together, these findings suggest that regulation on lipid biosynthesis in MG might exert therapeutic benefits for obstructive MGD and related MG atrophy (Table 2).

6. Morphological improvement under current clinical managements for MGD

Extensive evidence has confirmed that current physical therapies and pharmacological interventions may effectively alleviate the ocular morbidities and symptoms of MGD. However, morphological and structural changes of MG after treatments for MGD have not been clearly demonstrated, likely due to the lack of precise and objective tools for the clinical evaluation of the therapeutic effects, and any improvement remains controversial. A retrospective study of 78 eyes reported that MG dropout is decreased by 5% after 1-month of eyelid hygiene [80]. Another cohort study of 43 patients using morphometric analysis also observed an increase in visible MG structure 1-year after treatment by vectored thermal pulsation [81]. However, no increase has been detected regarding MG size in most clinical trials following warm compress and Lipiflow treatment [82]. Intraductal MG probing (MGP), a mechanical treatment for obstructive MGD that is thought to open and dilate the MG orifice by insertion of microprobes [83], has been reported to promote gland regrowth in a case series of 19 patients [84], while others studies showed no morphological improvement [85,86]. Intense pulse light, a therapy used in dermatology practice for years, has been introduced as a treatment for MGD in recent years. Using meibography and in vivo confocal microscopy, a cohort study of 35 patients found that IPL and eyelid hygiene both improved MG atrophy by 4–5%, while IPL changed the microstructure of MG with significant increase in the acinar diameter and unit density [87]. It has been proposed that the sustaining therapeutic benefits of IPL on MG microstructure was due to a photo-modulatory effect, which might promote the activity of MG acinar cells, however supportive evidence is still lacking. And 3% diquafosol treatment for 4 months has been shown by Arita et al., to effectively increase the MG area in patients diagnosed with obstructive MGD [88].

In recent years, a series of automated objective evaluation tools have been introduced to grade and quantify the MG morphology via infrared meibographs [89–94]. Deng et al. developed a fully automated multi-parametric MG analyzer that could greatly aid the diagnosis and grading of MGD [93]. Wang et al. using deep learning approaches via image segmentation achieved precise quantification of MG parameters such as atrophy percentage, gland length, gland width, gland tortuosity and ghost gland [92]. In addition, they reported an automatic approach for evaluation of MG atrophy using unsupervised feature learning [94]. These advanced automated MG analyzers might be further applied in clinical practice for managing MGD and evaluating the therapeutic improvement on MG structures. Considering the conflicting results among current clinical studies on the improvement of MG structure via a variety of therapies for MGD, future prospective longitudinal studies with longer follow-ups and accurate morphometric analysis should provide valuable insights regarding the reversal potentials of atrophic MG in MGD.

7. Conclusion and perspective

In recent years, wide application of genetic tools has greatly enhanced our knowledge regarding the search for MG stem/progenitor cells. It has been suggested that MG stem/progenitors are potentially a heterogeneous population residing in both the central ductal epithelium and the interfacial regions between the ductules and acini of MG. Furthermore, evidence suggests that under homeostatic conditions, there are two committed stem cell populations that give rise independently to proliferating ductal epithelium and meibocyte/acinar progenitor cells. Evidence also exists that under degenerating conditions that these two populations may be bipotent, giving rise to both duct and meibocytes, or that an alternative stem cell source can be recruited to regenerate the meibomian gland. Clearly, more in-depth studies are needed to confirm these possibilities and to more precisely identify and define the cellular dynamics and lineage diversity or MG during development, homeostasis and regeneration. This will clearly entail unraveling the specific biomarkers for the MG stem cell population and delineating the effects of age and disease on the stem cell survival and ability to renew progenitor cell populations, as well as uncover the effects of currently proposed therapeutic strategies and discover novel and more efficacious approaches. However, it should be noted that the current clinical therapies for MGD only exhibit minor or no improvement to the MG morphology and/or function, and that we need a major shift in our understanding of the molecular and regulatory mechanisms guiding MG function that could provide the platform necessary to finally address MGD and the problem of EDED.

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Abbreviations

ARMGD	age-related Meibomian gland dysfunction
EDED	evaporative dry eye disease
MG	Meibomian gland
MGD	Meibomian gland dysfunction

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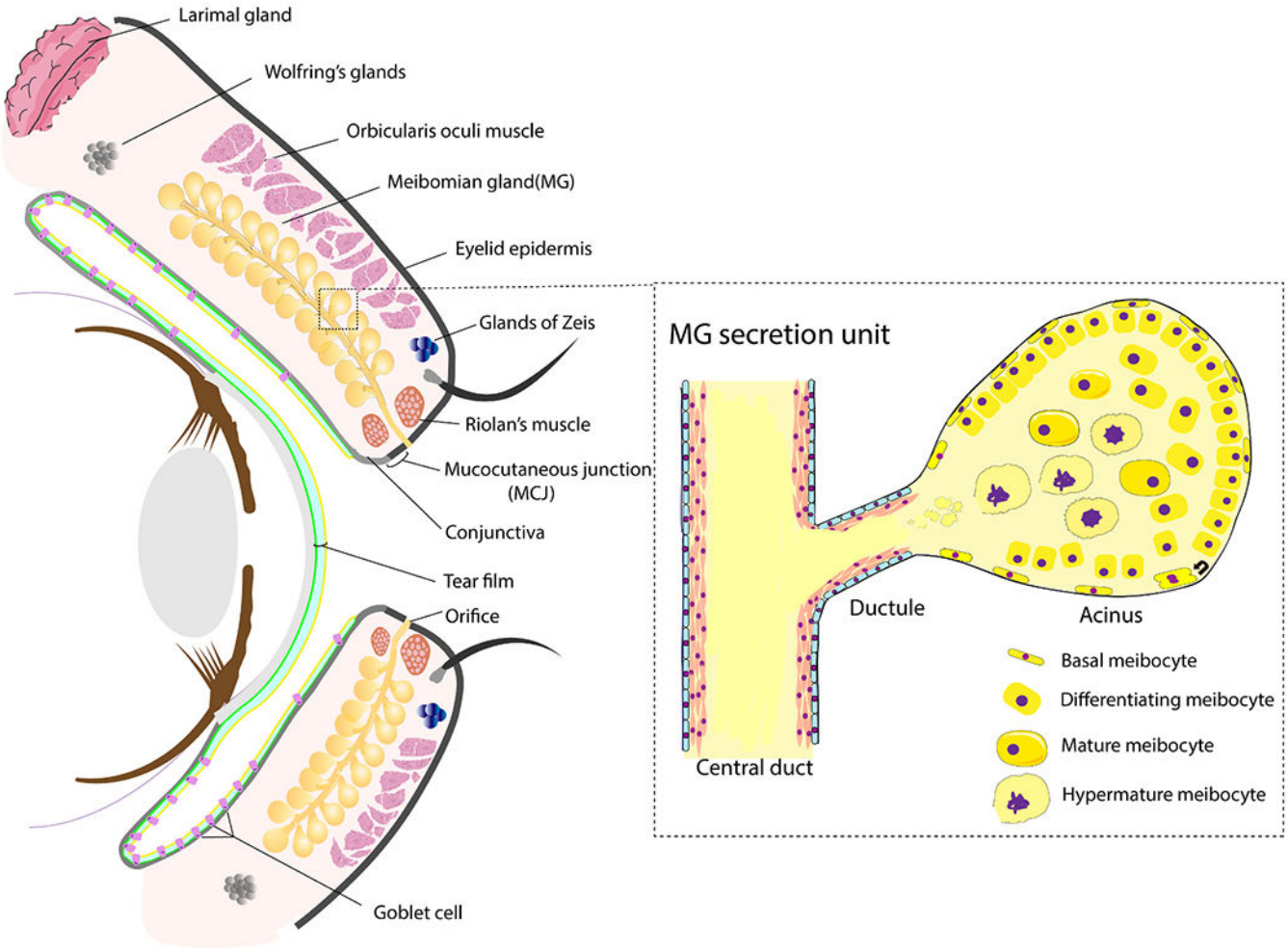


Fig. 1. The anatomy and secretion mode of the human meibomian glands.

Schematic illustration of the sagittal view of human eyelid and the secretion unit of the meibomian gland.

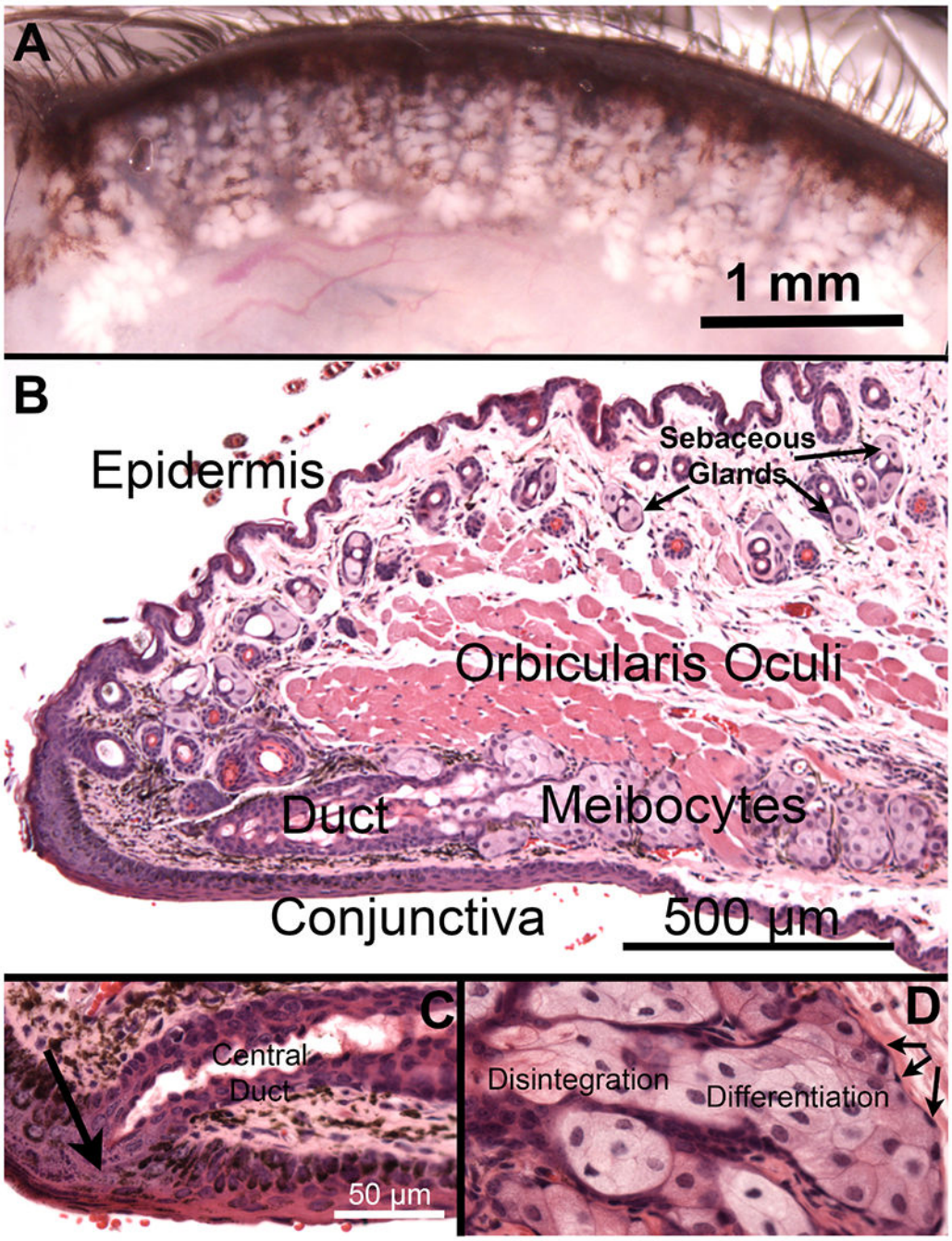


Fig. 2. Histology of the Mouse eyelid.

A. Reflected light meibography of the mouse upper eyelid showing a vertical row of meibomian glands. B. Sagittal histologic section of the mouse eyelid showing the meibomian gland with distal duct and more proximal secretory acini with meibocytes underlying the orbicularis oculi muscle and adjacent conjunctiva. C. Higher magnification of the central meibomian gland duct near the orifice (arrow). D. Higher magnification of a meibomian gland acini showing basal progenitor cells (arrows), and meibocytes undergoing differentiation and disintegration.

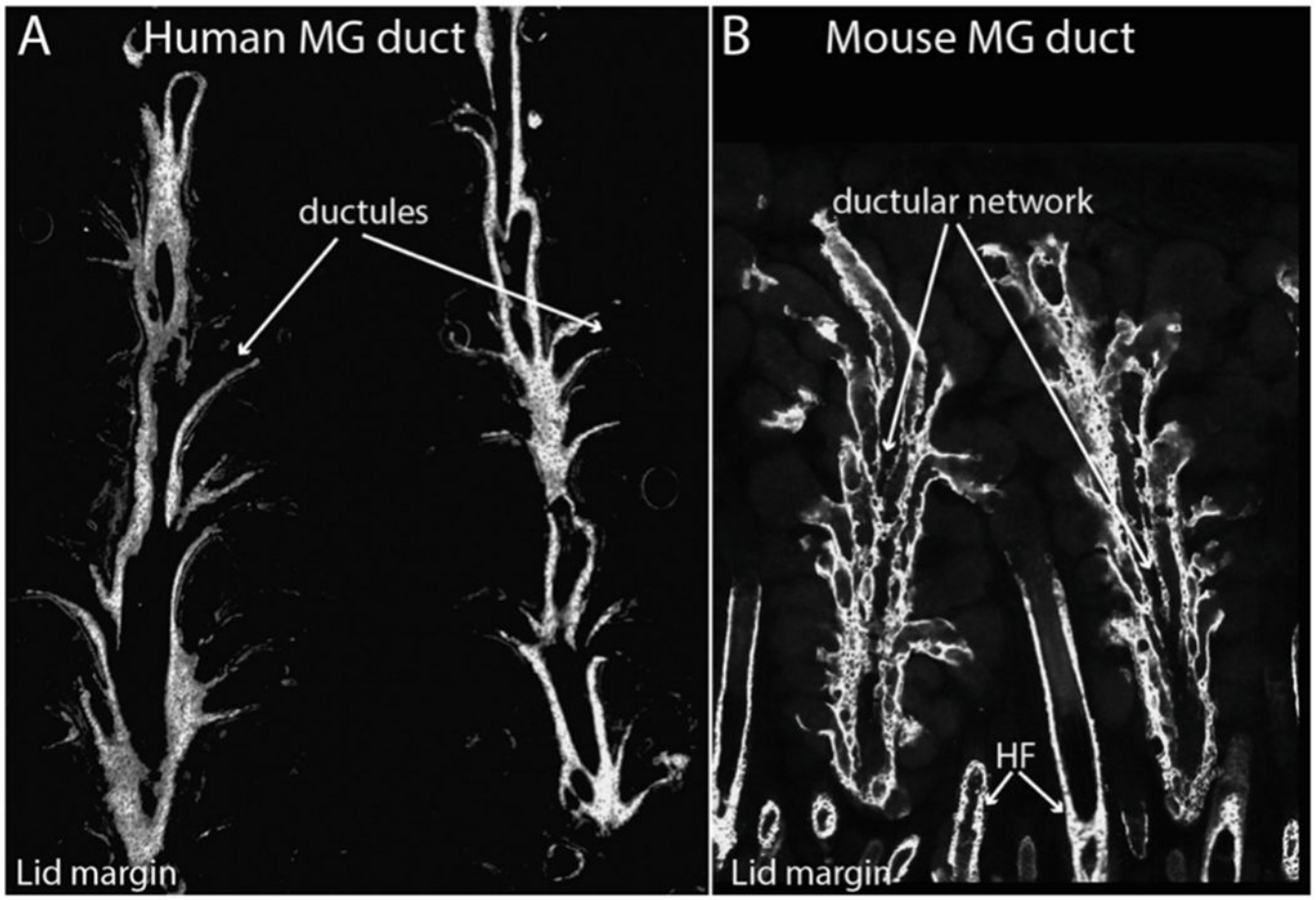
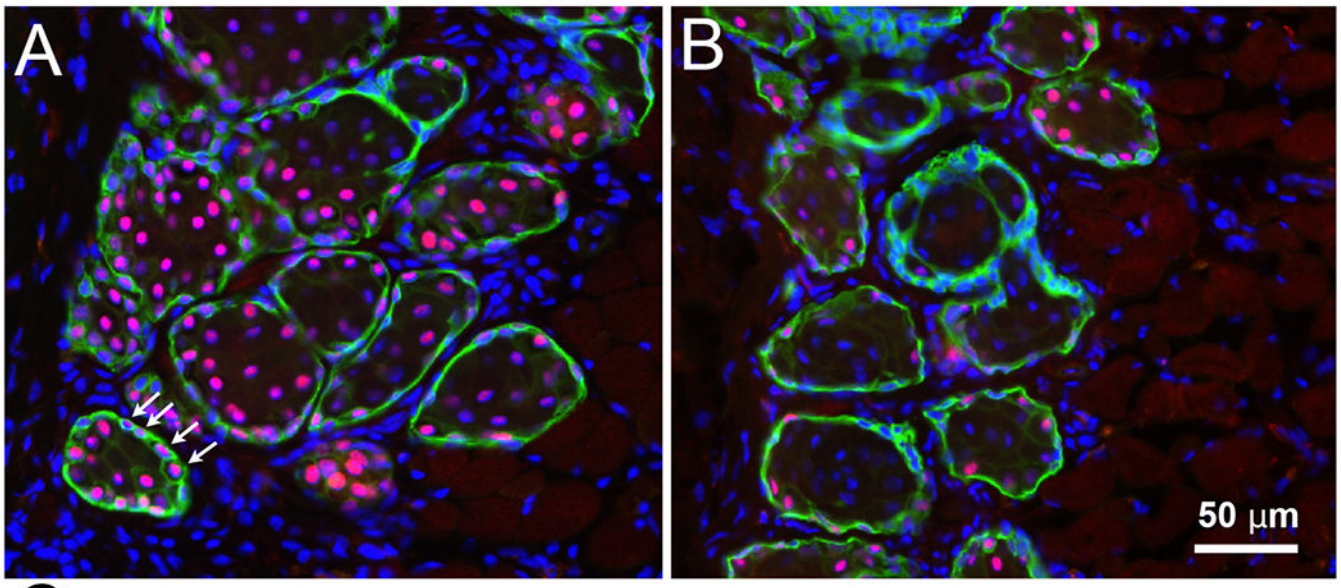


Fig. 3.

The distinct ductal systems of human and mouse meibomian glands outlined by Krt6a immunostaining. (A) The ductal compartment of human MG consists of a long central duct and dozens of short individual ductules. (B) The mouse MG exhibits highly intertwined ductular network which elongate and merge into the central duct cavity.



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Age	Mouse	Meibocyte Nuclei/100 μm			PPARγ⁺ Nuclei		
		Total	% Acinar	% Basal	% Total	% Acinar	% Basal
2 Month	791	11.23	38%	62%	36%	65%	29%
	792	11.14	31%	69%	45%	50%	44%
	793	10.08	32%	68%	35%	44%	33%
	Mean	**10.82**	**33%**	**67%**	**39%**	**53%**	**35%**
	SD	**0.64**	**4%**	**4%**	**6%**	**11%**	**7%**
1 Year	763	10.4	31%	69%	20%	20%	21%
	764	10.8	29%	71%	18%	24%	17%
	766	11.8	33%	67%	24%	18%	26%
	Mean	**11.0**	**31%**	**69%**	**20%**	**21%**	**21%**
	SD	**0.7**	**2%**	**2%**	**3%**	**3%**	**5%**
	P-Value	**0.73**	**0.36**	**0.38**	**0.01**	**0.01**	**0.05**

Fig. 4.

Meibocyte Krt5 and PPARγ co-localization. (A). Young (2 month old) mouse meibomian gland stained for Krt5 (Green), PPARγ (Red) and DAPI (Blue). Krt5 expression is limited to the meibocytes progenitor cell population in the basal compartment of the MG acinus, while differentiating meibocytes show intense PPARγ staining and are Krt5 negative. Note that some Krt5 positive, basal cells show variable staining for PPARγ, with nuclear size and PPARγ labeling intensity inversely proportional to Krt5 staining (arrows). (B) Old (1 year old) mouse meibomian gland showing markedly decreased PPARγ labeling of both basal and acinar meibocytes. (C) Quantification of PPARγ labeling showing significantly higher

number PPARγ positive cells in the basal and suprabasal compartment in young compared to old meibomian glands.

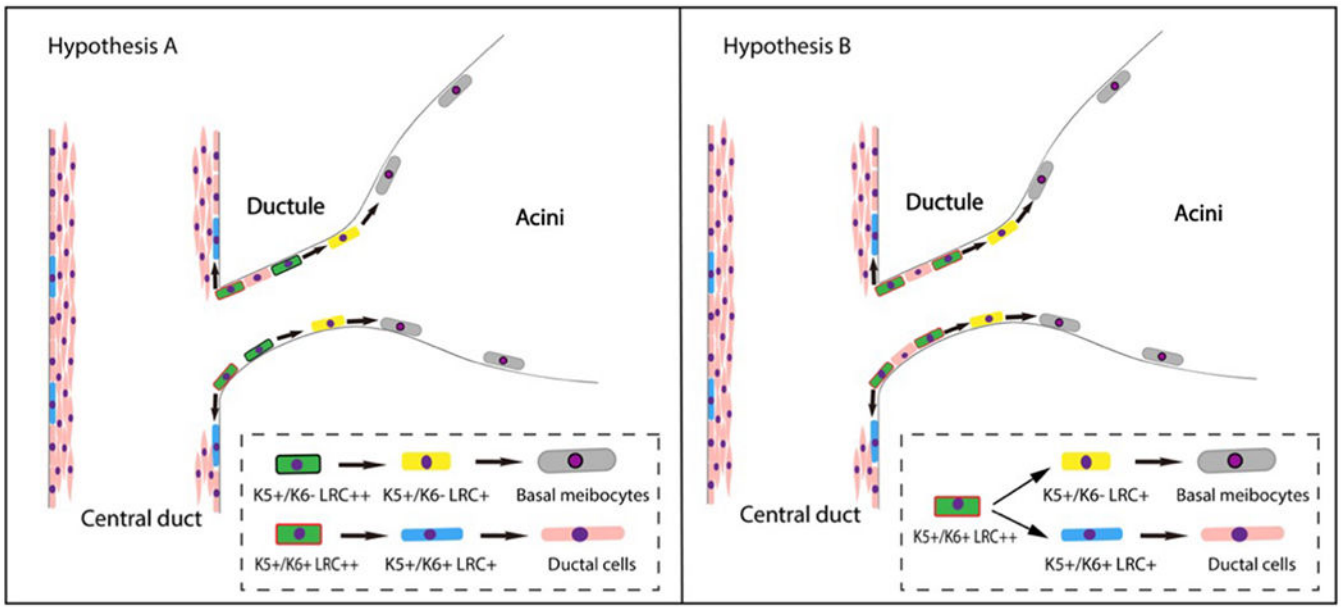

Fig. 5.

Schematic diagram of hypotheses about MG progenitors proposed by Parfitt et al. based on LRC experiment. Hypothesis A: The $K5^{+}/K6^{+}$ and $K5^{+}/K6^{-}$ unipotent progenitors independently give rise to ductal and acinar cells of MG. Hypothesis B: The $K5^{+}/K6^{+}$ bipotent progenitors maintain the renewal of the ductal and acinar cells of MG.

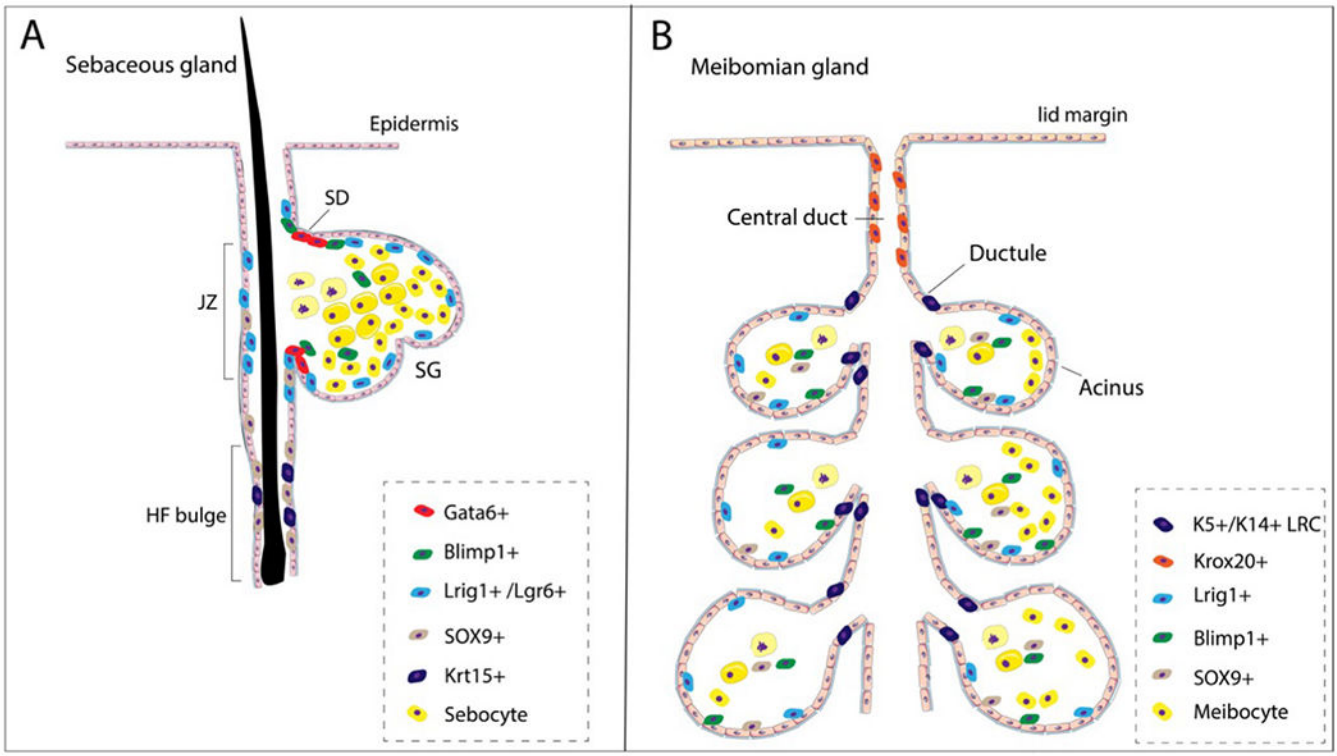

Fig. 6.

Putative biomarkers for stem/progenitor cells in SG and MG during adulthood. (A) Diagram of heterogeneous stem/progenitor compartments contributing to SG homeostasis. (B) Expression pattern of proposed MG stem/progenitor markers. SG, sebaceous gland, MG, meibomian gland, HF, hair follicle, SD, sebaceous duct, JZ, junction zone.

Table 1

Putative stem/progenitor cell markers investigated in the Meibomian gland.

Study	Markers							Expression pattern	Methods	Species
	K5	K14	Sox9	Blimp1	Lrig1	CD147	Krox20			
Geraint et al. (2015)	+		+	+				Quiescent and differentiated meibocytes	LR, IS	mice
Mauris et al. (2015)						+		Basal meibocytes	IS, PCR, *CD147*^{KO} model	human, mice
Geraint et al. (2016)	+	+		+				Terminal ends of the ductal epithelium	LT, LR	mice
Xie et al. (2018)					+			Basal meibocytes	IS, WB	human
Edem et al. (2021)							+	Suprabasal layer of the ductal epithelium	IS, LT, *Krox20*^{cKO} model	mice
Selikem et al. (2022)	+				+			Basal compartment of MG spheroids	iHMGECs organoids	human

Abbreviations: LR, Label-retaining study; IS, immunostaining; LT, Lineage-tracing study; WB, Western blot; iHMGECs, immortalized human meibomian gland epithelial cells.

Table 2

Novel therapeutic targets promoting the regrowth of meibomian gland in animal models.

Study	Drug	Target	Effects	Mechanism of action	Experimental model
Fan al. (2019)	PEDF-derived peptide 29-mer	Lrig1/ΔNp63+ basal cells	Increased MG area by 1.37-fold	Promoting basal cell proliferation	Aged C57BL/6 mice
Wang et al. (2022)	Periplocin	Na+/K + ATPase	Increased cell proliferation and lipid accumulation	Enhance proliferation and ΔNp63 basal cell activity via SRC signaling	Aged C57BL/6 mice, MMGECs
Sasaki et al. (2022)	NMN, NR	NAD+/3β-HSD	Enlarged MG by 1.14-fold and ameliorated the EDE signs	Improved proliferation and meibomian morphogenic factors- Wnt2 and Adamts17	Aged C57BL/6J mice
Ji et al. (2021)	SPT inhibitor-L-cyclo	Ceramide biosynthesis	Improved MG metabolism and morphology	Blocked abnormal cell differentiation, keratinization and reduced cell apoptosis	Pharmacological SCD1-deficiency mice
Chen et al. (2021)	Rosi-hydrogel	PPAR γ	Delayed aged related MG atrophy and attenuated the inflammation	Maintained expression of PPARγ and suppressed the NF-κB pathway	9-month-old mice
Ikeda et al. (2018)	Diquafosol	P2Y2 receptor	Improved meibum lipid droplets and reduced keratinization	Activation of P2Y2 receptors in MG acini	$Sod1^{-/-}$ mice
Ibrahim et al. (2017)	DHEA	Androgen receptor	Improved MG acinar area	Androgenic effect	Albino rats

Abbreviations: PEDF, pigment epithelium-derived factor; MMGECs, mouse meibomian gland epithelial cells; NMN, nicotinamide mononucleotide; NR, nicotinamide riboside; EDE, evaporative dry eye; SPT, serine palmitoyltransferase 1; Sod, Cu, Zn-superoxide dismutase-1; ROSI, Rosiglitazone; DHEA, Dehydroepiandrosterone.