



# Establishment and characterization of a canine sebaceous epithelial cell line derived from an eyelid mass

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**ABSTRACT.** Little is known about the pathological roles of sebaceous glands in canine skin diseases, as most examinations have been conducted with cultured human sebaceous epithelial cell lines. To our knowledge, there is no available canine sebaceous epithelial cell line. The purpose of this study was to establish a canine sebaceous epithelial cell line and characterize it. An eyelid mass in a dog was surgically resected for treatment, and it was histologically diagnosed as sebaceous epithelioma. Collected tissue was conducted for culture, and the growing epithelial-like cells were passaged. The cells showed continuous proliferation for over 6 months. After 40 passages, the cells were named CMG-1. Lipid droplets in the cytoplasm of CMG-1 cells were confirmed by Oil Red O staining. As reported in studies with human sebaceous epithelial cell lines, lipogenesis in CMG-1 cells was promoted by linoleic acid, whereas transforming growth factor- $\beta$  (TGF- $\beta$ ) suppressed it. Additionally, real-time PCR revealed that the expression levels of chemokines and cytokines, including CC chemokine ligand (CCL)-2, CCL-20, CXCL-10, Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), Interleukin (IL)-1 $\alpha$ , IL-1 $\beta$ , and IL-8, were significantly increased in CMG-1 cells following treatment with lipopolysaccharide. In conclusion, we successfully established a new canine sebaceous epithelial cell line. Our data indicated that lipogenesis and inflammatory responses were quantitatively evaluable in this cell line. CMG-1 cells could be useful for the pathological analysis of sebaceous gland diseases in dogs.

**KEY WORDS:** cell line, dog, meibomian gland, sebaceous epithelial cell, sebaceous gland

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Sebaceous glands are exocrine glands in the skin [26]. The alveolar structures in these sebaceous glands are lined by reserve cells, which differentiate into mature sebocytes and are finally excreted by holocrine secretion. The secreted oily and waxy material (sebum) contains triglycerides, fatty acids, wax esters, squalene, cholesterol esters, and cholesterol [6, 24, 25, 36]. Although most sebaceous glands are connected to hair follicles, they are also found in hairless areas, including the eyelids, oral epithelium, and ears [21, 42]. Meibomian glands are such “free sebaceous glands” which are located in the eyelids and have a cell physiology similar to the sebaceous glands located in the skin [14, 22, 30, 39].

Sebaceous gland dysfunction is involved in the pathogenesis of various human skin diseases such as acne vulgaris, seborrhea, and psoriasis [33, 42]. Sebum is associated with functional maintenance of the skin surface by controlling moisture balance and providing native immunity. Thus, low secretion of sebum leads to increased dryness and fragility of the skin [11]. In contrast, excessive sebum production is one of the major causes of acne vulgaris [42]. Because secretion of sebum is due to the progression of lipogenesis, numerous studies on the molecular mechanism of lipogenesis in sebaceous glands have been conducted [37, 40, 42]. Peroxisome proliferator-activated receptor (PPAR)  $\gamma$  has been reported to be a lipid-sensitive nuclear receptor that regulates sebocyte differentiation and lipogenesis [13]. The transforming growth factor (TGF)- $\beta$  signaling pathway has been suggested to suppress lipogenesis and maintain the undifferentiated condition of human sebocytes [21]. Some therapeutic candidates for human sebaceous gland diseases are those which modulate the signal pathway of lipogenesis, including PPAR $\gamma$  and TGF- $\beta$  [11, 19, 21]. Perturbations of sebaceous glands in dogs may cause diseases such as seborrheic dermatitis, sebaceous adenitis, and meibomian gland disorder. However, the physiological and pathological molecular mechanisms of lipogenesis in canine sebaceous glands, including the involvement of PPAR $\gamma$ , are not yet fully understood.

To date, the involvement of bacteria in the development of human sebaceous gland diseases has been documented. Recently, genome-wide analysis revealed that Toll-like receptor (TLR) 1/2 and TLR4-activation upregulated chemokines, including CC

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chemokine ligand (CCL)-2, CCL-20, and C-X-C motif chemokine ligand 10 (CXCL10), in human sebocytes [32]. Meanwhile, some studies revealed that proinflammatory cytokines such as interleukin (IL)-1 $\alpha$ , IL-1 $\beta$ , IL-8, and tumor necrosis factor (TNF)- $\alpha$  in human sebaceous epithelial cells were upregulated by lipopolysaccharide (LPS) treatment [23, 37, 40]. It was also reported that human sebocytes recruit neutrophils, monocytes, and T cells in an IL-8-dependent manner and induce the differentiation of naïve T cells into T helper (Th) 17 cells via secretion of IL-1 $\beta$  [20]. On the other hand, there have not yet been any studies investigating the molecular response of canine sebocytes under pathological conditions induced by bacteria.

Cultured cell lines are effective tools for molecular analysis. To date, some human sebaceous epithelial cell lines derived from the skin or eyelid have been established [4, 17, 18, 29, 42]. SZ95 and SEB-1 cells were immortalized using the Simian virus 40 T large antigen [29, 42]. Seb-E6E7 cells and SEBO662 cells were reported to be immortalized through the expression of E6 and E7 oncoproteins of human papillomavirus 16 [4, 18]. These cell lines were all derived from human skin. Besides, HMGEC cells, a cell line immortalized with retroviral human telomerase reverse transcriptase derived from a human eyelid, have also been established [17]. In contrast, the culture of canine sebaceous epithelial cells has not yet been reported in the literature. It is widely known that the components of sebaceous lipids as well as the structures of sebaceous glands differ among species [24, 25]. For instance, squalene was reported to be absent in canine sebaceous lipids [36], even though it constituted approximately 12–20% of human sebaceous lipids [25]. Therefore, sebocytes derived from dogs and not from humans should be used for the investigation of canine sebaceous gland diseases.

In the present study, we aimed to establish a canine sebaceous epithelial cell line. Additionally, lipogenesis and inflammatory responses of the established cell line were investigated for characterization.

## MATERIALS AND METHODS

### Dog

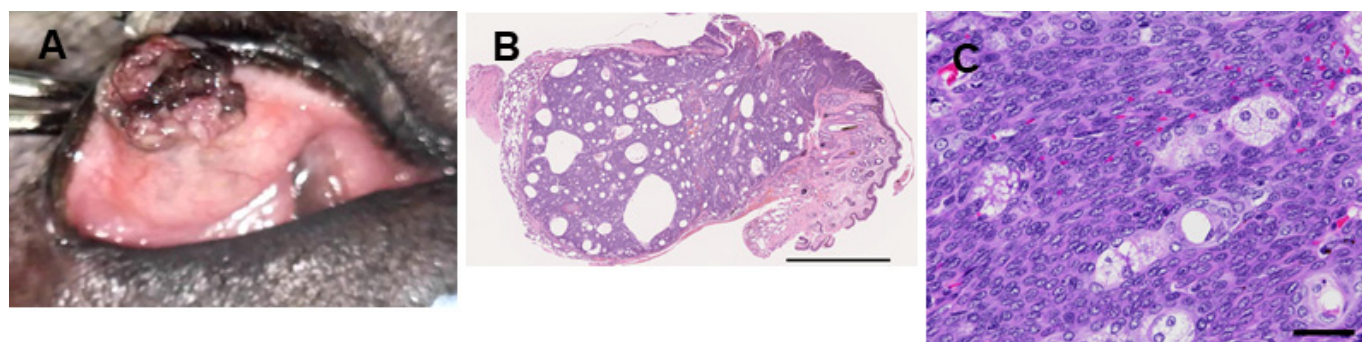
A dog with an eyelid mass measuring 10.0  $\times$  5.5  $\times$  4.0 mm and located slightly outside the center of the right upper eyelid with swelling of the conjunctiva was included in the present study (Fig. 1A). Surgical techniques used for treatment rather than for research purposes were used. All clinical examinations and treatments were performed after the dog's owner gave informed and written consent. The research's use of clinical of the dog's data and clinical samples was also agreed upon by the owner.

### Surgical treatment and histopathological examination

The eyelid mass was surgically resected under general anesthesia with a common surgical procedure. The resected mass was cut into half. One tissue half was used for cell culture, while the other remaining tissue half was used for histological examination. For histological analysis, hematoxylin and eosin staining was performed.

### Cell culture

Cell culture was performed according to a previous study with some modifications [2]. The collected and halved tissue was washed with sterile phosphate-buffered saline (PBS). A central region of the mass was cut aseptically with autoclaved scissors and forceps and then minced in a sterile plastic dish. The minced fragments were incubated in RPMI1640 (Fujifilm Wako Chemicals, Tokyo, Japan) and supplemented with 10% fetal bovine serum (FBS) (Biosera, Nuaille, France) and antibiotics at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. After a 3-day culture, proliferating epithelial-like cells with a small number of fibroblasts were observed. To exclude fibroblasts, the epithelial-like cells were picked up with a micropipette under a microscope after washing with PBS. The collected cells were then seeded in a new plastic flask. The epithelial-like cells were passaged at 70%–80% confluence. For passage, the cells were washed with Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free PBS and then detached from the flasks by incubation



**Fig. 1.** Photographs of the eyelid mass. (A) The photograph shows the gross appearance of the eyelid mass. (B) The well-delineated mass occupied the dermis and subcutis with mildly asymmetric expansile growth. Scale bars are equal to 2 mm. Hematoxylin and eosin. (C) The mass is composed of lobules and islands of polyhedral to short spindle-shaped neoplastic cells with slight atypia. Occasional differentiation of the tumor cells to sebocytes and rare tubular structures mimicking sebaceous ducts support the diagnosis of a sebaceous epithelioma. Scale bars are equal to 40  $\mu$ m. Hematoxylin and eosin.

with 2.5 g/l of trypsin and 1 mmol/l of ethylenediaminetetraacetic acid (EDTA) (Nakarai Tesque, Kyoto, Japan) at 37°C for 5 min. The cells were washed and suspended in culture medium. Cell viability and number were assessed using a trypan blue dye exclusion test. The cell suspension was diluted 1:10 with culture medium, after which the cells were seeded in a new plastic flask.

#### Chromosome number

Chromosome counts were obtained from CMG-1 cells at the 45th passage. After 48 hr of incubation in culture medium, semiconfluent and actively dividing cells were incubated in 0.5 µg/ml KaryoMAX Colcemid (Thermo Fisher Scientific, Waltham, MA, USA) diluted in culture medium for 2 hr at 37°C. Harvested cells were pelleted out and treated with hypotonic solution (1% NaCl and 0.55% KCl in H<sub>2</sub>O, 4:1) for 25 min prior to fixation in methanol-acetic acid (3:1). The suspension of fixed cells was dropped onto microscope slides. Following air-drying, the cells were stained with 10% Giemsa (Sigma, St. Louis, MO, USA) for 30 min. Finally, the chromosome numbers of 1 × 10<sup>2</sup> cells were observed and counted under a microscope.

#### Lipid droplet analysis

For Oil Red O staining, the cultured cells were washed twice with Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free PBS and incubated overnight at room temperature with 4% paraformaldehyde. After washing with distilled water, the cells were treated with 60% isopropanol for 1 min and stained with Oil Red O solution at 37°C for 20 min. The cells were then washed twice with 60% isopropanol and once with water.

For the detection of lipid droplets, Lipi-Green (Dojindo, Kumamoto, Japan) was also used according to the manufacturer's instructions. CMG-1 cells at the 45th passage were incubated with culture medium for 48 hr before the examination. For induction or suppression of lipogenesis, the cells were incubated with 100 µM of linoleic acid (LA) (Nacalai Tesque, Tokyo, Japan) or 5 ng/ml of human recombinant TGF-β1 (PeproTech, Rocky Hill, NJ, USA) for 24 hr. After washing with PBS, the cultured cells were incubated with Lipi-Green working solution (0.1 µM in PBS) for 30 min at 37°C. Fluorescence microscopy and flow cytometry (BD LSRFortessa X-20, Becton, Dickinson and Co., Franklin Lakes, NJ, USA) were used for the detection of Lipi-Green-positive cells. The mean fluorescence intensity (MFI) of CMG-1 cells, including both Lipi-Green-positive cells and Lipi-Green-negative cells, was measured. After flow cytometric analysis, 5 × 10<sup>3</sup> cells were detected in each measurement.

#### Real-time polymerase chain reaction

CMG-1 cells at the 45th passage were incubated with culture medium for 48 hr before the examination. After incubation with 100 µM of LA, 5 ng/ml of TGF-β, and 1 µg/ml of LPS from *Escherichia coli* O111:B4 (Cat. No. L4391) or medium alone for 6 hr, total RNA was extracted from the cells (5 × 10<sup>5</sup> cells) using a NucleoSpin Plus kit (Takara Bio, Kusatsu, Japan). The extracted RNA was then reverse-transcribed into cDNA using a PrimeScript II 1st strand cDNA synthesis kit (Takara Bio).

Real-time PCR was performed with TB Green Premix Ex Taq II (Takara Bio) in the presence of 0.2 µM each of the forward and reverse primers for canine PPARγ, canine C-C motif chemokine ligand (CCL)-2, canine CCL-20, canine C-X-C motif chemokine ligand (CXCL)-10, canine TNF-α, canine IL-1α, canine IL-1β, canine IL-8, or canine β-actin (Supplementary Table 1). The PCR amplification consisted of pre-denaturation (95°C, 10 sec), 40 cycles of denaturation (95°C, 10 sec), annealing, and extension (60°C, 30 sec). Fluorescence intensity was measured in real-time during the extension steps using the QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific). Relative expression levels of the target gene were normalized to the endogenous reference (β-actin) and calculated by 2<sup>-ΔΔCT</sup>. All values were standardized to a value in a sample of the medium alone. The efficiencies of PCR for inflammatory cytokines and those for β-actin were approximately equal (Supplementary Table 1).

#### Statistical analysis

Statistical significance was analyzed using an analysis of variance (ANOVA) followed by a Tukey test or Student's *t*-test. The analysis was performed using EZR, which is a statistical software for R version 3.5.2 [12]. *P* values of <0.05 were considered statistically significant.

## RESULTS

#### Histological analysis of resected eyelid mass

The well-delineated mass occupied the dermis and subcutis, showing mildly asymmetric expansile growth without invasion of the surrounding normal tissue (Fig. 1B). The resected mass was composed of lobules and islands of polyhedral to short spindle-shaped neoplastic cells with slight atypia (Fig. 1C). The tumor cells had scant, pale, and eosinophilic cytoplasm, as well as mildly anisokaryotic, ovoid, euchromatic nuclei with conspicuous nucleoli. Some tumor cells had differentiated into sebocytes, while the majority (more than 90%) of the tumor cells resembled reserve cells. Tubular structures mimicking the sebaceous duct were occasionally present within the mass. Mitotic figures were only observed in the neoplastic reserve cells, which were counted to be 20 upon inspection of 10 high-power fields. The connective tissue stroma was scant. The tumor cells did not exhibit any observable vascular invasion. The bottom of the mass was surrounded by numerous foamy macrophages. Based on the above-mentioned findings, the mass was diagnosed as sebaceous epithelioma, which is considered a low-grade malignant neoplasm of sebaceous and modified sebaceous glands in animals.

### Cell culture

Epithelial-like cells isolated from the eyelid mass showed continuous cell growth for over 6 months. Cell viability was maintained at above 95% throughout the culture period. After 40 passages, the cells were named CMG-1. CMG-1 cells proliferated exponentially, with their doubling time being  $11.8 \pm 1.2$  hr (Fig. 2). The cells' chromosome numbers ranged from 46 to 83, with the modal number being 78 (Fig. 3). The cells proliferated to form irregularly shaped colonies with some piled up cells (Fig. 4A). They had polygonal shapes at less than 80% confluence but were hexagonal at 100% confluence (Fig. 4B).

In previous reports, sebocytes were identified by lipophilic staining with Oil Red O, Nile Red, or Sudan IV [29, 41]. Oil Red O staining revealed that CMG cells had small lipid droplets in their cytoplasm (Fig. 4B). Lipi-Green, which is a fluorescent reagent that is highly selective to lipid droplets, also accumulated in the cytoplasmic vesicles of CMG-1 cells (Fig. 4C).

### Lipogenesis in CMG-1 cells

LA treatment was reported to enhance lipogenesis in human sebaceous epithelial cells, whereas TGF- $\beta$  was found to suppress lipogenesis [11, 21, 26]. We evaluated whether CMG-1 cells respond to these stimuli. Oil Red O staining showed that lipid droplets in CMG-1 cells were increased by LA treatment and decreased by TGF- $\beta$  treatment (Fig. 5A–C). Flow cytometry was used for the quantitative analysis of Lipi-Green-positive lipid droplets (Fig. 5D, 5E). LA treatment significantly enlarged the Lipi-Green-positive cell proportion in CMG-1 cells, whereas TGF- $\beta$  treatment significantly reduced them (medium alone,  $44.20 \pm 4.18\%$ ; LA treatment,  $68.30 \pm 0.56\%$ ; TGF- $\beta$  treatment,  $24.83 \pm 0.56\%$ ). Moreover, MFI of CMG-1 cells were significantly increased by LA treatment but decreased by TGF- $\beta$  treatment (medium alone,  $3.39 \pm 0.47$ ; LA treatment,  $6.85 \pm 0.31$ ; TGF- $\beta$  treatment,  $1.80 \pm 0.08$ ).

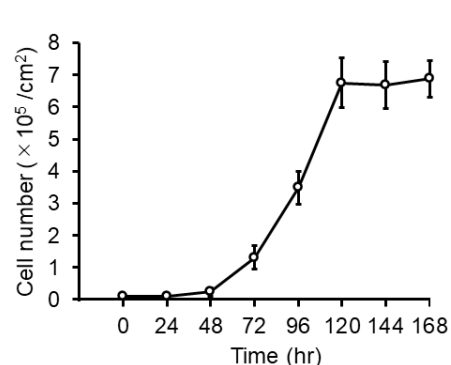
PPAR $\gamma$  has been reported to regulate lipogenesis in human sebaceous epithelial cells derived from the skin and the eyelid [11, 13, 19, 21, 38]. As shown in Fig. 6, real-time PCR showed that LA treatment significantly increased the expression level of PPAR $\gamma$  in CMG-1 cells, whereas TGF- $\beta$  treatment had no effect (medium alone,  $0.75 \pm 0.43$ ; LA treatment,  $19.98 \pm 8.44\%$ ; TGF- $\beta$  treatment,  $0.76 \pm 0.45$ ).

### Expression of inflammation-related genes in CMG-1 cells

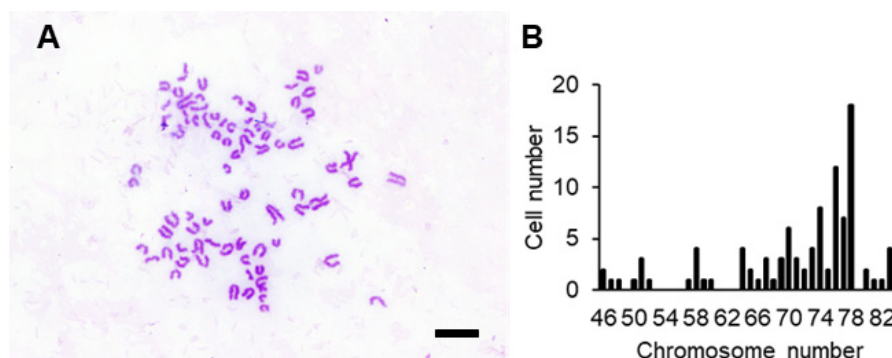
Finally, we investigated whether CMG-1 cells respond to bacterial components and produce chemokines and cytokines (Fig. 7). LPS incubation significantly elevated expression levels of CCL-2 (medium alone,  $0.68 \pm 0.28$ ; LPS treatment,  $15.59 \pm 1.60$ ), CCL-20 (medium alone,  $2.00 \pm 0.87$ ; LPS treatment,  $175.66 \pm 10.44$ ), CXCL-10 (medium alone,  $1.63 \pm 0.55$ ; LPS treatment,  $33.79 \pm 3.77$ ), TNF- $\alpha$  (medium alone,  $0.93 \pm 0.07$ ; LPS treatment,  $9.44 \pm 1.27$ ), IL-1 $\alpha$  (medium alone,  $0.99 \pm 0.01$ ; LPS treatment,  $1.80 \pm 0.13$ ), IL-1 $\beta$  (medium alone,  $1.17 \pm 0.15$ ; LPS treatment,  $1.48 \pm 0.09$ ), and IL-8 (medium alone,  $1.04 \pm 0.05$ ; LPS treatment,  $1.56 \pm 0.03$ ).

## DISCUSSION

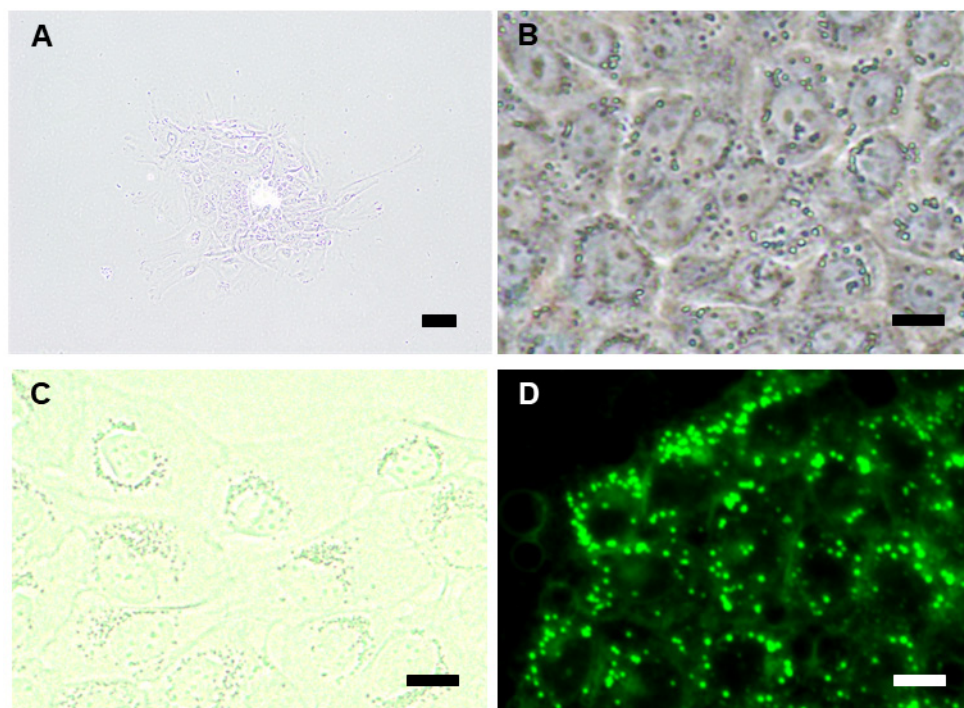
In this study, we successfully established a canine sebaceous epithelial cell line. CMG-1 cells were derived from a canine sebaceous epithelioma located in the eyelid. The shapes of cultured CMG-1 cells were quite similar to the reserve cells observed in the resected mass. Additionally, our data demonstrated that CMG-1 cells had lipid droplets in the cytoplasm similar to those seen in human sebaceous epithelial cell lines [17, 29, 41]. These observations support that CMG-1 cells were indeed a sebaceous epithelial cell line.



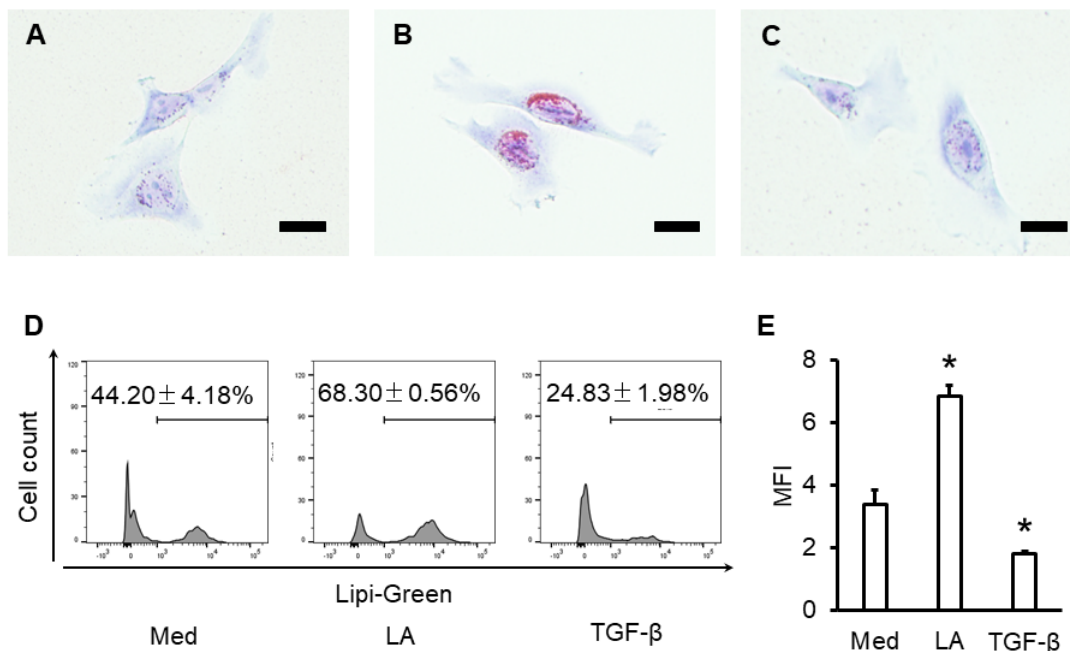
**Fig. 2.** Proliferation curve of CMG-1 cells. Proliferation activity was evaluated using a trypan blue exclusion test. Cells were suspended in culture medium at  $1 \times 10^4$  cells/ml and cell numbers were counted every 24 hr. The data are presented as means  $\pm$  SD of three individual experiments.



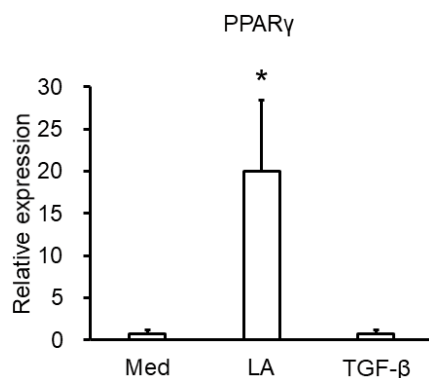
**Fig. 3.** Chromosome numbers of CMG-1 cells. (A) The photograph represents Giemsa-stained metaphase chromosomes from CMG-1 cells. The scale bar is equal to  $10 \mu\text{m}$ . (B) A graph representing the number of chromosomes found in CMG-1 cells.



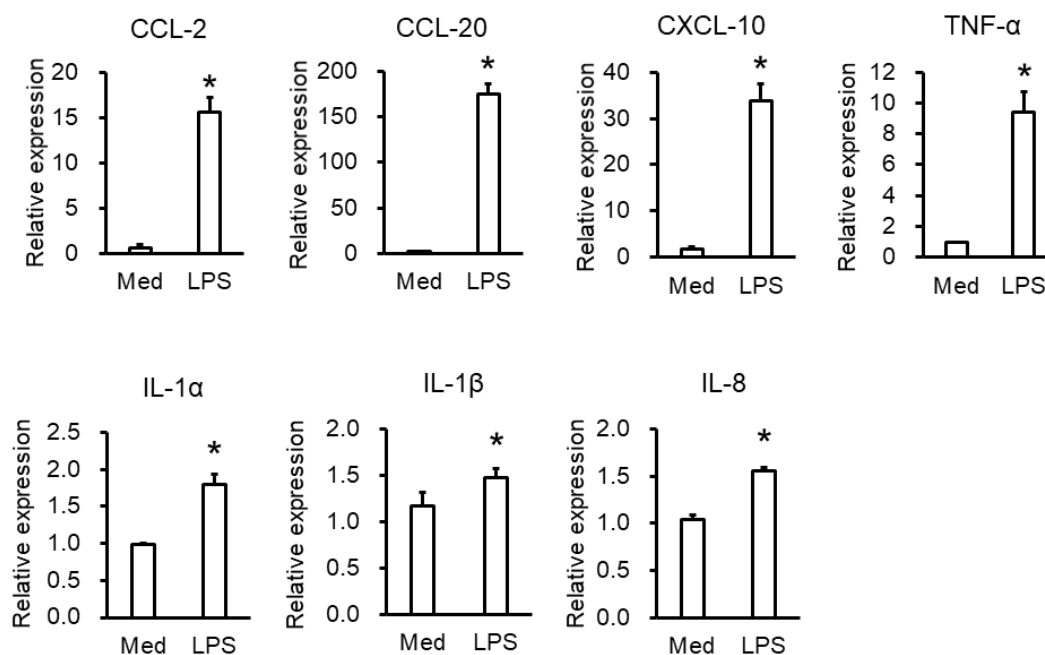
**Fig. 4.** Photographs of CMG-1 cells. The photographs represent CMG-1 cells which were (A, B) unstained, (C) stained with Oil Red O, and (D) treated with Lipi-Green. The fluorescein of Lipi-Green was detected by a fluorescence microscope (excitation laser: 488 nm; emission filter: 500–550 nm). The scale bars in the photographs are equal to 10  $\mu$ m.



**Fig. 5.** Lipogenesis in CMG-1 cells. Photographs of CMG-1 cells after 24 hr-culture with (A) medium alone, (B) 100  $\mu$ M of linoleic acid (LA), (C) and 5 ng/ml of transforming growth factor- $\beta$  (TGF- $\beta$ ). Lipid droplets were stained with Oil Red O while the cells were counterstained with hematoxylin. The scale bars in the photographs are equal to 20  $\mu$ m. (D) Lipid droplets in CMG-1 cells were quantitatively analyzed with flow cytometry. The cells were treated with Lipi-Green and then analyzed by a flow cytometer (excitation laser: 488 nm; emission filter: 500–560 nm). The numbers in the histograms show Lipi-Green-positive CMG-1 cells after 24 hr-culture with medium alone, 100  $\mu$ M of LA, or 5 ng/ml of TGF- $\beta$ . (E) The graph indicates the mean fluorescence intensity (MFI) of Lipi-Green-treated CMG-1 cells after 24 hr culture with medium alone, 100  $\mu$ M of LA, or 5 ng/ml of TGF- $\beta$ . The data are presented as means  $\pm$  SD of three individual experiments. Med indicates medium alone. \* $P$ <0.05 vs. Med.



**Fig. 6.** Expression of Peroxisome proliferator-activated receptor (PPAR)  $\gamma$  in CMG-1 cells. Real-time PCR was performed to detect the expression level of PPAR $\gamma$ . Total RNA of CMG-1 cells were extracted after 6 hr-culture with medium alone, 100  $\mu$ M of linoleic acid (LA), or 5 ng/ml of transforming growth factor- $\beta$  (TGF- $\beta$ ). Relative expression levels of the target gene were normalized to a value of  $\beta$ -actin and calculated by  $2^{-\Delta\Delta CT}$ . Values were standardized to Med. The data are presented as means  $\pm$  SD of three individual experiments. Med indicates medium alone. \* $P < 0.05$  vs. Med.



**Fig. 7.** Expression of inflammation-related genes in CMG-1 cells. Real-time PCR was performed to detect the expression levels of CC chemokine ligand (CCL)-2, CCL-20, C-X-C motif chemokine ligand 10 (CXCL10), Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), Interleukin (IL)-1 $\alpha$ , IL-1 $\beta$ , and IL-8. Total RNA of CMG-1 cells was extracted after 6 hr-culture with or without 1  $\mu$ g/ml of lipopolysaccharide (LPS). Relative expression levels of the target gene were normalized to a value of  $\beta$ -actin and calculated by  $2^{-\Delta\Delta CT}$ . Values were standardized to Med. The data are presented as means  $\pm$  SD of three individual experiments. Med indicates medium alone. \* $P < 0.05$  vs. Med.

Histological analysis diagnosed the resected eyelid mass to be a sebaceous epithelioma. As far as the authors know, there is no histological grading system for sebaceous epithelioma to specifically characterize the outcome or metastatic behavior of the neoplasm. Histological differentiation between sebaceous epithelioma and sebaceous carcinoma is usually straightforward [10]. Sebaceous epitheliomas show a preponderance of neoplastic reserve cells and mitotic activity is only found in reserve cell components [10]. On the other hand, sebaceous carcinomas are composed of less differentiated and more atypical neoplastic epithelial cells with cytoplasmic lipid droplets, with mitotic activity seen in both sebocytes and reserve cells [10]. Metastasis of sebaceous epitheliomas to distant sites has seldom been reported in animals [5]. Although the successfully established cell line might be linked to malignancy of the resected mass, the given case was free of clinical findings suggestive of metastasis to regional lymph nodes or any distant sites. Further examinations should be performed to clarify the relationship between the grade of malignancy and the culturable characteristics of canine sebaceous epitheliomas.

Our results revealed that lipogenesis and PPAR $\gamma$  upregulation can easily be induced by LA treatment in CMG-1 cells, which is similar to that seen in human sebocytes. Meanwhile, although TGF- $\beta$  treatment suppressed the accumulation of lipid droplets in CMG-1 cells, it did not suppress the expression level of PPAR $\gamma$ . Sebaceous lipogenesis, which leads to the accumulation of lipid droplets and sebum secretion, represents a major step in the terminal differentiation of sebocytes [10, 37]. Thus, induction and suppression of lipogenesis or PPAR $\gamma$  upregulation in human sebaceous epithelial cell lines were performed to investigate the molecular mechanisms underlying sebocyte differentiation [10, 15, 17, 22, 24]. Our results suggested that CMG-1 cells could be used for the analysis of the molecular mechanisms underlying the differentiation of canine sebocytes.

The involvement of certain bacteria in the development of human sebaceous gland diseases has been widely studied [1, 30, 36, 37], but the underlying molecular mechanisms are still not fully understood. The induction of proinflammatory cytokines, such as IL-1 $\alpha$ , IL-1 $\beta$ , IL-8, and TNF- $\alpha$ , after LPS treatment has been observed in human sebocytes [23, 37, 40]. As far as the authors know, no studies on whether canine sebaceous epithelial cells respond to LPS treatment have been conducted. In this study, we demonstrated the similar results using the established cell line. The expression level of TNF- $\alpha$  was especially elevated after LPS treatment. TNF- $\alpha$  was reported to promote lipogenesis in SZ95 cells and thus suggested as a possible therapeutic target for the control of seborrhea and acne vulgaris [7]. Therefore, LPS-induced TNF- $\alpha$  might be promote lipogenesis after exposure to LPS in canine epithelial cells.

The results of this study showed that the expression levels of chemokines in CMG-1 cells were greatly elevated by LPS treatment. CCL-2 and CCL-20 are chemoattractants for monocytes and lymphocytes, respectively [27, 35]. CXCL-10 chemoattracts a wide range of cells, including macrophages, dendritic cells, NK cells, and NKT cells [31]. There are few reports describing the relationship between these chemokines and sebocytes [28]. However, it is commonly known that lymphocytes and macrophages infiltrate into the lesions of canine sebaceous adenitis [3, 9, 16]. Additionally, a recent study demonstrated that epithelium-derived CCL20 recruited the skin-resident innate lymphoid cells, which limited sebocyte growth and regulated the microbiota [15]. Further studies using CMG-1 cells might uncover the role of chemokines in the pathological or physiological condition of canine sebaceous glands.

CMG-1 cells were derived from tumor tissues. Compared with the cell lines derived from non-tumor cells, CMG-1 cells could have modifications in their molecular mechanisms. In fact, aneuploidy, which is defined as the alteration of chromosome number, was detected in CMG-1 cells and may have led to transcriptome and proteome change [8, 34]. Thus, the phenomena exhibited by CMG-1 cells should be confirmed through comparison with non-tumor cells or tissues.

In conclusion, this study was able to establish and characterize a canine epithelial sebaceous cell line for the first time, which was named as CMG-1. These CMG-1 cells could be a useful tool for the pathological analysis of sebaceous gland diseases in dogs.

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## REFERENCES

1. Adalsteinsson, J. A., Kaushik, S., Muzumdar, S., Guttman-Yassky, E. and Ungar, J. 2020. An update on the microbiology, immunology and genetics of seborrheic dermatitis. *Exp. Dermatol.* **29**: 481–489. [Medline] [CrossRef]
2. Azakami, D., Shibutani, H., Dohi, M., Takasaki, M., Ishioka, K., Mori, A., Momota, Y., Bonkobara, M., Washizu, T., Michishita, M., Hatakeyama, H., Ogasawara, S. and Sako, T. 2011. Establishment and characterization of canine rhabdomyosarcoma cell line CMS-C. *J. Vet. Med. Sci.* **73**: 1105–1108. [Medline] [CrossRef]
3. Bardagi, M., Fondevila, D., Zanna, G. and Ferrer, L. 2010. Histopathological differences between canine idiopathic sebaceous adenitis and canine leishmaniosis with sebaceous adenitis. *Vet. Dermatol.* **21**: 159–165. [Medline] [CrossRef]
4. Barrault, C., Dichamp, I., Garnier, J., Pedretti, N., Juchaux, F., Deguercy, A., Agius, G. and Bernard, F. X. 2012. Immortalized sebocytes can spontaneously differentiate into a sebaceous-like phenotype when cultured as a 3D epithelium. *Exp. Dermatol.* **21**: 314–316. [Medline] [CrossRef]
5. Bettini, G., Morini, M., Mandrioli, L., Capitani, O. and Gandini, G. 2009. CNS and lung metastasis of sebaceous epithelioma in a dog. *Vet. Dermatol.* **20**: 289–294. [Medline] [CrossRef]
6. Camera, E., Ludovici, M., Galante, M., Sinagra, J. L. and Picardo, M. 2010. Comprehensive analysis of the major lipid classes in sebum by rapid resolution high-performance liquid chromatography and electrospray mass spectrometry. *J. Lipid Res.* **51**: 3377–3388. [Medline] [CrossRef]
7. Choi, J. J., Park, M. Y., Lee, H. J., Yoon, D. Y., Lim, Y., Hyun, J. W., Zouboulis, C. C. and Jin, M. 2012. TNF- $\alpha$  increases lipogenesis via JNK and PI3K/Akt pathways in SZ95 human sebocytes. *J. Dermatol. Sci.* **65**: 179–188. [Medline] [CrossRef]
8. Dürbaum, M. and Storchová, Z. 2016. Effects of aneuploidy on gene expression: implications for cancer. *FEBS J.* **283**: 791–802. [Medline] [CrossRef]
9. Frazer, M. M., Schick, A. E., Lewis, T. P. and Jazic, E. 2011. Sebaceous adenitis in Havanese dogs: a retrospective study of the clinical presentation and incidence. *Vet. Dermatol.* **22**: 267–274. [Medline] [CrossRef]
10. Goldschmidt, M. H. and Goldschmidt, K. H. 2016. Epithelial and melanocytic tumors of the skin. pp. 88–141. *In: Tumors in Domestic Animals*, John Wiley & Sons, Inc., Hoboken.
11. Hong, I., Lee, M. H., Na, T. Y., Zouboulis, C. C. and Lee, M. O. 2008. LXR $\alpha$  enhances lipid synthesis in SZ95 sebocytes. *J. Invest. Dermatol.* **128**: 1266–1272. [Medline] [CrossRef]
12. Kanda, Y. 2013. Investigation of the freely available easy-to-use software 'EZR' for medical statistics. *Bone Marrow Transplant.* **48**: 452–458. [Medline] [CrossRef]
13. Kim, S. W., Xie, Y., Nguyen, P. Q., Bui, V. T., Huynh, K., Kang, J. S., Brown, D. J. and Jester, J. V. 2018. PPAR $\gamma$  regulates meibocyte differentiation and lipid synthesis of cultured human meibomian gland epithelial cells (hMGEC). *Ocul. Surf.* **16**: 463–469. [Medline] [CrossRef]
14. Knop, E., Knop, N., Millar, T., Obata, H. and Sullivan, D. A. 2011. The international workshop on meibomian gland dysfunction: report of the subcommittee on anatomy, physiology, and pathophysiology of the meibomian gland. *Investig. Ophthalmology Vis. Sci.* **52**: 1938.

15. Kobayashi, T., Voisin, B., Kim, D. Y., Kennedy, E. A., Jo, J. H., Shih, H. Y., Truong, A., Doebel, T., Sakamoto, K., Cui, C. Y., Schlessinger, D., Moro, K., Nakae, S., Horiuchi, K., Zhu, J., Leonard, W. J., Kong, H. H. and Nagao, K. 2019. Homeostatic control of sebaceous glands by innate lymphoid cells regulates commensal bacteria equilibrium. *Cell* **176**: 982–997. [\[Medline\]](#) [\[CrossRef\]](#)
16. Linek, M., Boss, C., Haemmerling, R., Hewicker-Trautwein, M. and Mecklenburg, L. 2005. Effects of cyclosporine A on clinical and histologic abnormalities in dogs with sebaceous adenitis. *J. Am. Vet. Med. Assoc.* **226**: 59–64. [\[Medline\]](#) [\[CrossRef\]](#)
17. Liu, S., Hatton, M. P., Khandelwal, P. and Sullivan, D. A. 2010. Culture, immortalization, and characterization of human meibomian gland epithelial cells. *Invest. Ophthalmol. Vis. Sci.* **51**: 3993–4005. [\[Medline\]](#) [\[CrossRef\]](#)
18. Lo Celso, C., Berta, M. A., Braun, K. M., Frye, M., Lyle, S., Zouboulis, C. C. and Watt, F. M. 2008. Characterization of bipotential epidermal progenitors derived from human sebaceous gland: contrasting roles of c-Myc and  $\beta$ -catenin. *Stem Cells* **26**: 1241–1252. [\[Medline\]](#) [\[CrossRef\]](#)
19. Mastrofrancesco, A., Ottaviani, M., Cardinali, G., Flori, E., Briganti, S., Ludovici, M., Zouboulis, C. C., Lora, V., Camera, E. and Picardo, M. 2017. Pharmacological PPAR $\gamma$  modulation regulates sebogenesis and inflammation in SZ95 human sebocytes. *Biochem. Pharmacol.* **138**: 96–106. [\[Medline\]](#) [\[CrossRef\]](#)
20. Mattii, M., Lovász, M., Garzorz, N., Atenhan, A., Quaranta, M., Lauffer, F., Konstantinow, A., Küpper, M., Zouboulis, C. C., Kemeny, L., Eyerich, K., Schmidt-Weber, C. B., Töröcsik, D. and Eyerich, S. 2018. Sebocytes contribute to skin inflammation by promoting the differentiation of T helper 17 cells. *Br. J. Dermatol.* **178**: 722–730. [\[Medline\]](#) [\[CrossRef\]](#)
21. McNairn, A. J., Doucet, Y., Demaue, J., Brusadelli, M., Gordon, C. B., Uribe-Rivera, A., Lambert, P. F., Bouez, C., Breton, L. and Guasch, G. 2013. TGF $\beta$  signaling regulates lipogenesis in human sebaceous glands cells. *BMC Dermatol.* **13**: 2. [\[Medline\]](#) [\[CrossRef\]](#)
22. Miyazaki, M., Man, W. C. and Ntambi, J. M. 2001. Targeted disruption of stearyl-CoA desaturase1 gene in mice causes atrophy of sebaceous and meibomian glands and depletion of wax esters in the eyelid. *J. Nutr.* **131**: 2260–2268. [\[Medline\]](#) [\[CrossRef\]](#)
23. Nagy, I., Pivarcsi, A., Kis, K., Koreck, A., Bodai, L., McDowell, A., Seltmann, H., Patrick, S., Zouboulis, C. C. and Kemény, L. 2006. Propionibacterium acnes and lipopolysaccharide induce the expression of antimicrobial peptides and proinflammatory cytokines/chemokines in human sebocytes. *Microbes Infect.* **8**: 2195–2205. [\[Medline\]](#) [\[CrossRef\]](#)
24. Nikkari, T. 1974. Comparative chemistry of sebum. *J. Invest. Dermatol.* **62**: 257–267. [\[Medline\]](#) [\[CrossRef\]](#)
25. Picardo, M., Ottaviani, M., Camera, E. and Mastrofrancesco, A. 2009. Sebaceous gland lipids. *Dermatoendocrinol* **1**: 68–71. [\[Medline\]](#) [\[CrossRef\]](#)
26. Schneider, M. R. and Zouboulis, C. C. 2018. Primary sebocytes and sebaceous gland cell lines for studying sebaceous lipogenesis and sebaceous gland diseases. *Exp. Dermatol.* **27**: 484–488. [\[Medline\]](#) [\[CrossRef\]](#)
27. Shi, L., Wang, J., Ding, N., Zhang, Y., Zhu, Y., Dong, S., Wang, X., Peng, C., Zhou, C., Zhou, L., Li, X., Shi, H., Wu, W., Long, X., Wu, C. and Liao, W. 2019. Inflammation induced by incomplete radiofrequency ablation accelerates tumor progression and hinders PD-1 immunotherapy. *Nat. Commun.* **10**: 5421. [\[Medline\]](#) [\[CrossRef\]](#)
28. Smith, K. R. and Thiboutot, D. M. 2008. Thematic review series: skin lipids. Sebaceous gland lipids: friend or foe? *J. Lipid Res.* **49**: 271–281. [\[Medline\]](#) [\[CrossRef\]](#)
29. Thiboutot, D., Jabara, S., McAllister, J. M., Sivarajah, A., Gilliland, K., Cong, Z. and Clawson, G. 2003. Human skin is a steroidogenic tissue: steroidogenic enzymes and cofactors are expressed in epidermis, normal sebocytes, and an immortalized sebocyte cell line (SEB-1). *J. Invest. Dermatol.* **120**: 905–914. [\[Medline\]](#) [\[CrossRef\]](#)
30. Toffoli, B., Gilardi, F., Winkler, C., Soderberg, M., Kowalczyk, L., Arsenijevic, Y., Bamberg, K., Bonny, O. and Desvergne, B. 2017. Nephropathy in Pparg-null mice highlights PPAR $\gamma$  systemic activities in metabolism and in the immune system. *PLoS One* **12**: e0171474. [\[Medline\]](#) [\[CrossRef\]](#)
31. Tomita, K., Freeman, B. L., Bronk, S. F., LeBrasseur, N. K., White, T. A., Hirsova, P. and Ibrahim, S. H. 2016. CXCL10 mediates macrophage, but not other innate immune cells-associated inflammation in murine nonalcoholic steatohepatitis. *Sci. Rep.* **6**: 28786. [\[Medline\]](#) [\[CrossRef\]](#)
32. Töröcsik, D., Kovács, D., Póliska, S., Szentkereszty-Kovács, Z., Lovász, M., Hegyi, K., Szegedi, A., Zouboulis, C. C. and Stähle, M. 2018. Genome wide analysis of TLR1/2- and TLR4-activated SZ95 sebocytes reveals a complex immune-competence and identifies serum amyloid A as a marker for activated sebaceous glands. *PLoS One* **13**: e0198323. [\[Medline\]](#) [\[CrossRef\]](#)
33. Wikramanayake, T. C., Borda, L. J., Miteva, M. and Paus, R. 2019. Seborrheic dermatitis-Looking beyond Malassezia. *Exp. Dermatol.* **28**: 991–1001. [\[Medline\]](#) [\[CrossRef\]](#)
34. Williams, B. R. and Amon, A. 2009. Aneuploidy: cancer's fatal flaw? *Cancer Res.* **69**: 5289–5291. [\[Medline\]](#) [\[CrossRef\]](#)
35. Wunderlich, C. M., Ackermann, P. J., Ostermann, A. L., Adams-Quack, P., Vogt, M. C., Tran, M. L., Nikolajev, A., Waisman, A., Garbers, C., Theurich, S., Mauer, J., Hövelmeyer, N. and Wunderlich, F. T. 2018. Obesity exacerbates colitis-associated cancer via IL-6-regulated macrophage polarisation and CCL-20/CCR-6-mediated lymphocyte recruitment. *Nat. Commun.* **9**: 1646. [\[Medline\]](#) [\[CrossRef\]](#)
36. Yoon, J. S., Nishifuji, K., Ishioroshi, S., Ide, K. and Iwasaki, T. 2013. Skin lipid profiling in normal and seborrheic shih tzu dogs. *Vet. Dermatol.* **24**: 84–9. [\[Medline\]](#) [\[CrossRef\]](#)
37. Zákány, N., Oláh, A., Markovics, A., Takács, E., Aranyász, A., Nicolussi, S., Piscitelli, F., Allarà, M., Pór, Á., Kovács, I., Zouboulis, C. C., Gertsch, J., Di Marzo, V., Bíró, T. and Szabó, T. 2018. Endocannabinoid tone regulates human sebocyte biology. *J. Invest. Dermatol.* **138**: 1699–1706. [\[Medline\]](#) [\[CrossRef\]](#)
38. Zhang, M. F., Cai, X. L., Jing, K. P., Pi, X. X., Liao, P. Y., Li, S. J., Wen-Li, Cai, C.-C., Quan, J.-H., Fan, Y.-M. and Ge-Shi. 2018. Differentiation model establishment and differentiation-related protein screening in primary cultured human sebocytes. *BioMed Res. Int.* **2018**: 1–10.
39. Zhang, S. D., He, J. N., Niu, T. T., Chan, C. Y., Ren, C. Y., Liu, S. S., Qu, Y., Chong, K. L., Wang, H. L., Tao, J. and Pang, C. P. 2017. Bacteriological profile of ocular surface flora in meibomian gland dysfunction. *Ocul. Surf.* **15**: 242–247. [\[Medline\]](#) [\[CrossRef\]](#)
40. Zouboulis, C. C., Picardo, M., Ju, Q., Kurokawa, I., Töröcsik, D., Bíró, T. and Schneider, M. R. 2016. Beyond acne: Current aspects of sebaceous gland biology and function. *Rev. Endocr. Metab. Disord.* **17**: 319–334. [\[Medline\]](#) [\[CrossRef\]](#)
41. Zouboulis, C. C., Seltmann, H., Neitzel, H. and Orfanos, C. E. 1999. Establishment and characterization of an immortalized human sebaceous gland cell line (SZ95). *J. Invest. Dermatol.* **113**: 1011–1020. [\[Medline\]](#) [\[CrossRef\]](#)
42. Zouboulis, C. C., Picardo, M., Ju, Q., Kurokawa, I., Töröcsik, D., Bíró, T. and Schneider, M. R. 2016. Beyond acne: Current aspects of sebaceous gland biology and function. *Rev. Endocr. Metab. Disord.* **17**: 319–334. [\[Medline\]](#) [\[CrossRef\]](#)