Establishment of functional epithelial organoids from human lacrimal glands

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Research

Keywords: Organoid, Lacrimal gland, Dry eye disease, Sjogren syndrome

DOI: https://doi.org/10.21203/rs.3.rs-56825/v2

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Abstract

**Background:** Tear deficiency due to dysfunction of the lacrimal gland (LG) is one of the major causes of dry eye disease (DED). Therefore, LG stem cell-based therapies have been extensively reported to regenerate injured lacrimal tissue, but the number of stem cells in the LG tissue is pretty low, and 2D long-term cultivation results in the reduction of the differentiation capacity of stem cells. Whereas, 3D LG organoids could be an alternative for a DED therapeutic method, because it is capable of prolonged growth while maintaining the characteristics of the LG tissue. In this study, we developed LG organoids and applied them as cell therapeutics.

**Methods:** Digested cells from human LG tissue were mixed with Matrigel and cultured in five different media modified from human prostate/salivary organoid culture media. After organoid formation, the growth, expression of specific markers and histological characters were analyzed to prove the LG organoids. And then, the secretory function of LG organoids was confirmed by calcium influx or proteomics after pilocarpine treatment. To explore the curability of developed organoids, finally, mouse-derived lacrimal gland organoids were fabricated and transplanted into mouse lacrimal tissue with DED.

**Results:** The histological features and specific marker expression of LG organoids were similar to normal LG tissue. In the pilocarpine treated LG organoid, internal Ca2+ ions and β -hexosaminidase as known a lysosomal protein in tear fluid was increased. Also, the secreted proteins from pilocarpine treated lacrimal organoid were identified through proteomics. More than 70% of the identified proteins were proven to exosome through GO analysis. These results indicate that our developed organoid has a reactivity to pilocarpine, which shows the function of the lacrimal gland. Additionally, we developed LG organoids from Sjogren's syndrome patients (SS) and confirmed that their histological features similar to that of SS-derived LG tissue. Finally, we confirmed that organoids were well engrafted in the lacrimal tissue at 2 weeks after transplantation of mouse LG organoid.

**Conclusion:** This current study demonstrate that our established lacrimal gland organoids resemble characteristics of normal lacrimal gland tissue and could be used as cell therapy for patients with dry eye syndrome.

**Background**

Dry eye disease (DED) is a continously disabling disease occurring in 11-22% of population, withabout 17% of them developing water deficient dry eye [1,2]. A previous study has reported that a severe type of DES could occur in about half of patients presenting structural and functional damage in the lacrimal gland [3]. DEDis a multifactorial life-long debilitating disorder mainly caused by functional disturbances in the lacrimal gland [4]. Causes of glandular dysfunction range from deficiency and loss of tear film integrity, deterioration of the lacrimal gland, to death of the secretory epithelial cells affected by hormonal imbalance, environmental changes, and autoimmune pathologies, leading to DED, a chronic condition [4].
DED has also been defined as a multifactorial disease of the ocular surface characterized by the lack of tear film stability. It is accompanied by ocular symptoms in which unstable tear film, hyperosmolarity, ocular surface inflammation and damage, and neurosensory abnormalities play pathologic roles [5]. Its etiologies include causes secondary to systemic autoimmune disorders such as Sjogren's syndrome, rheumatoid arthritis, systemic lupus erythematosus, and so on that are bothersome to doctors and patients [6-10]. In conventional treatment, artificial tear eye drops are mainly used to moisturize the ocular surface and offer additional lubrication [11]. To address symptoms of chronic inflammation, anti-inflammation and topical immunosuppression agents could be considered [12]. However, their persistent use is limited due to side effects. [5,12]. Other regenerative strategies have recently been introduced to make a qualitative advance in the field of DED management [13].

Interestingly, recent studies have demonstrated the habitation of stem cells in exocrine glands such as salivary [14], pancreas [15,16], prostate [17], and breast [18,19]. However, in the case of lacrimal gland, reports on the residence of stem cells in mouse lacrimal gland [20,21] and human lacrimal gland are fewer than those in other exocrine glands.

Contrarily to lacrimal gland cultures growing a monolayer in order of preference on Matrigel, collagen, and HAM in two to three weeks, the formation of spheroids including a mixed population of stem cells and differentiated cells has been reported in salivary gland cultures [22] and prostate spheres [23]. A single study has been conducted on spheroidal aggregation of rabbit lacrimal gland cells grown in a microgravity environment of a rotary cell culture system [24]. Salivary spheres including stem cells could rescue function of the gland when they are transplanted into animal models of radiation-induced dry mouth [22].

To the best of our knowledge, there are few reports of organoid establishment using stem cells in human lacrimal gland. Under certain culture conditions, suspension 3D cultures of human “lacrispheres” could be maintained and propagated for 3-4 weeks. In addition, lacrimal spheres could secrete quantifiable levels of tear protein into conditioned media [23]. Lacrimal gland epithelial cells can make ‘spherules' with channel like connections. Measurements for levels of secretary IgA, lysozyme, and lactoferrin have explained the ductal origin in conditioned media [4].

Regarding structural and functional loss of the lacrimal gland, one promising option for managing this condition could be replacement of the gland and restoration of its function through cell therapy [23]. Organoid based therapy containing stem cells would help recover and differentiate into functionally competent cells which could lead to regeneration of damaged tissues.

There are increasing evidences for the prevalence of stem-like cells in the lacrimal gland of mice [21,25] that contribute to reconstruction of injured gland. We attempted to establish cultures of human lacrimal gland organoid and evaluate stem cell components by immunophenotyping, clonal assays, and real-time functional assay. We aimed to rebuild an organoid presenting 3D lacrimal gland
tissue functional unit incorporating different cell types. It could provide a premise of treatment option for severe DED models including Sjogren's disease.

**Methods**

**Cell isolation and organoid formation from human lacrimal gland tissue**

Normal lacrimal gland tissues were obtained from patients with eye-related disease and harvested from non-damaged regions without inflammation. Tissues were chopped and washed with advanced DMEM/F12 (Gibco, Carlsbad, CA, USA) containing 1% penicillin-streptomycin (Welgene, Gyeongsan-si, Korea) and then enzymatically digested with advanced DMEM/F12 containing 0.125 mg/ml dispase II (Wako, Richmond, VA, USA), 0.1 mg/ml DNase I (MilliporeSigma, Burlington, MA, USA), 0.125 mg/ml collagenase II (Gibco, Carlsbad, CA, USA), and 1% penicillin-streptomycin for 1 hour at 37°C with shaking (150rpm). After digestion, the supernatant was passed through a 70 µm cell strainer (SPL, Pocheon-si, Gyeonggi-do, Korea) and pelleted by spinning down. The pellet was resuspended in culture media and mixed with Matrigel (Corning, Corning, NY, USA) at a ratio of 1:1 (v:v), plated onto a 48-well plate at a density of 1X10⁴ per well, and incubated with 5% CO₂ at 37°C for 10 min for polymerization of matrices. Lacrimal gland organoids were cultured in five different media modified from human prostate and salivary organoid culture medium [26,27]. Components of each medium are listed in Supplementary Table 1. The culture medium was changed every 2-3 days.

To confirm the origin of cells forming the organoid, EpCAM-positive epithelial lineage cells were sorted using the MACS method (Miltenyibiotec, 130-042-201). Briefly, single cells from lacrimal tissue were incubated with anti-EpCAM (Santacruz, sc-59906) for 1 hour at 4°C, washed with MACS buffer, and then incubated anti-mouse IgG Microbeads (Miltenyibiotec, 130-048-402) for 30 min at 4°C. After washing with PBS, EpCAM negative cells were passed through a MACS column. EpCAM positive cells in the MACS column were isolated, washed, and then cultured in Matrigel.

**Histology and immunofluorescence**

Tissues and organoids were washed with D-PBS (Welgene, Gyeongsan-si, Korea), fixed with 4% paraformaldehyde (Bio-solution, Seoul, Korea) for 30 min, and embedded in paraffin. Paraffin sections of 6 µm in thickness were deparaffinized in xylene and hydrated in a graded series of ethanol. These samples were then stained with H&E, Alcian blue, PAS staining kit (Abcam, Cambridge, MA, USA), and Masson's trichrome staining kit (Dako, Santa Clara, CA, USA) according to their respective manufacturer's protocol. For immunofluorescence analysis, fixed samples were cryoprotected by immersing in PBS containing 30% sucrose and 0.1% sodium azide at 4°C. The cryoprotected samples were embedded in optimal cutting temperature (OCT, Sakura) compound, rapidly frozen in liquid nitrogen, and stored at -80°C until use. Sections (4 µm in thickness) of the frozen block were pre-blocked with 5% normal horse serum (Vector) in Tris-buffered saline (Welgene, Gyeongsan-si, Korea) for 2 hours at room temperature (RT) and incubated with primary antibody at 4°C overnight. After washing with PBS,
sections were incubated with secondary antibody for 2 hours at RT. For nuclear staining, Hoechst 33342 (MilliporeSigma, Burlington, MA, USA, 1 ug/ml) treatment was performed for 20 min. Primary antibodies used for immunostaining included Aquaporin5 (Abcam, Cambridge, MA, USA), α-SMA (Biolegend, San Diego, CA, USA), Vimentin (Cell signaling, Danvers, MA, USA), Lysozyme (Diagnostic biosystems, Pleasanton, CA, USA), E-cadherin (Santa Cruz Biotechnology, Dallas, TX, USA), anti-BrdU (Novus, Centennial, CO, usa), and Ki67 (Abcam, Cambridge, MA, USA). Secondary antibodies (Thermo Fisher Scientific, Waltham, MA, USA) used included Alexa Fluor 488 goat anti-rabbit IgG, Alexa Fluor 594 goat anti-mouse IgG, and Alexa Fluor 594 goat anti-rat IgG.

**Total RNA isolation and quantitative RT-PCR**

Total RNAs were from isolated tissues or organoids using MagListo™ 5M Cell Total RNA Extraction Kit (Bioneer, Daejeon Metropolitan City, Korea) following the manufacturer’s protocol. Then 1 μg of RNA was used to synthesize cDNA using PrimeScript™ RT Master Mix (TaKaRa, Kyoto City, Japan). Quantitative RT-PCR was performed with a Thermal Cycler Dice® Real-Time System III (TaKaRa, Kyoto City, Japan) using SYBR® Premix Ex Taq™ II (TaKaRa, Kyoto City, Japan). Sequences of PCR primers are listed in Supplementary Table 2. PCR experiments were carried out in triplicate.

**Calcium flux assay with Fluo-4**

Mobilization of Ca2+ to the cytoplasm was detected using a Fluo-4 Calcium Imaging Kit (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer’s protocol. Briefly, organoids were treated with Fluo-4 AM for 15 min at 37°C followed by incubation at RT for 15 min. After washing with PBS, organoids were stimulated with pilocarpine of 1 μg/ml (MilliporeSigma, Burlington, MA, USA). Calcium signaling was then observed using a Nikon Eclipse Ti2 microscope (Nikon, Tokyo, Japan).

**β-Hexosaminidase assay**

To demonstrate the secretory function of lacrimal gland organoids, lysosomal enzyme N-acetyl-β-glucosaminidase (NAG), also known as α-galactosidase B, in organoid cultured medium was detected using a NAG assay kit (MilliporeSigma, Burlington, MA, USA) following the manufacturer’s protocol. Briefly, organoids were incubated in serum-free DMEM/F12 for 2 hours, treated with pilocarpine (1 ug/ml), and then incubated at 37°C in a 5% CO2 incubator for 24 hours. The medium was collected at 2 hours and 24 hours after pilocarpine treatment and analyzed for NAG catalytic activity with a NAG assay kit. Reaction product was detected colorimetrically at 405 nm using a microplate reader (Multiskan GO, Thermo Fisher Scientific, Waltham, MA, USA).

**Transmission electron microscope analysis**

To detect secretory proteins from organoids, transmission electron microscope (TEM) analysis was performed. Briefly, cultured organoids were washed with D-PBS and fixed with 2% glutaraldehyde-paraformaldehyde in 0.1M phosphate buffer (PB, pH7.4) for 12hr. After washing with 0.1M PB, samples were post-fixed with 1% OsO4 dissolved in 0.1 M PB for 2 h, dehydrated in an ascending gradual series
(50-100%) of ethanol, infiltrated with propylene oxide, and embedded with a Poly/Bed 812 kit (Polysciences). After pure fresh resin embedding and polymerization at 65°C in an electron microscope oven (DOSAKA) for 24 h, Poly/Bed embedded samples were cut into about 70 nm thick sections and stained with 6% uranyl acetate and lead citrate (Fisher) for contrast staining. These sections were cut with a Leica EM UC-7 (Leica Microsystems) equipped with a diamond knife (Diatome) and transferred onto copper and nickel grids. All thin sections were observed with a TEM (JEOL) at an acceleration voltage of 80kV.

**Proteomic analysis for secretome of lacrimal gland organoids**

To identify secreted proteins by pilocarpine in lacrimal gland organoids, the culture medium was harvested after 2 hours of pilocarpine treatment and analyzed by proteomics [28]. In brief, proteins (200 ug) in the medium were digested using filter-aided sample preparation (FASP) method with centrifugal filters (Millipore). After desalting samples with a Sep-Pak® Vac 1cc C18 cartridge (Waters), peptides were collected, purified, and quantified for LC-MS/MS analysis. LC-MS/MS assay was performed using a Dionex Ultimate 3000 HPLC coupled with a Q Exactive™ Hybrid Quadrupole-Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Raw MS/MS data were quantified using MaxQuant (Max Planck Institute) and classified by GO (Gene Ontology) analysis. T-test P-value of <0.05 and Fold-change (>2, <2) were applied to determine differential expression protein (DEP) between control and pilocarpine treated groups.

**Mouse dry eye model and organoid transplantation**

Eight-week-old male C57BL/6 mice (Koatech, Pyeongtaek, Korea) or C57BL/6- Tg(CAG-EGFP)131Osbg/LeySopJ (CAG-EGFP) mice (Nihon SLC) were used as dry eye disease model or for manipulation of lacrimal gland organoids, respectively. The experimental protocol for animal use was reviewed and approved by CHA University Institutional Animal Care and Use Committee. Lacrimal gland tissue was obtained from eGFP-Tg mouse. Organoids were formed and cultured following the same method used for human organoids. To create an inflammation-induced dye eye model, 15 µl of ConA (Concanavalin A, 10 mg/ml in PBS, MilliporeSigma, Burlington, MA, USA) was injected into the extra-orbital gland of wild type mouse lacrimal tissue. The same volume of PBS was injected for the control group. At 7 days after ConA injection, cell clumps from GFP expressed organoids (1 X 104 cells/15µl in advanced DMEM/F12/Matrigel) were injected into the extra-orbital gland space (Fig. 5). After 2 weeks, mouse lacrimal gland tissues were harvested for immunofluorescence analysis.

**Statistical analysis**

Statistically significant differences were analyzed by Student's t-test of one-way analysis of variance (ANOVA) with post hoc test Tukey for multiple comparisons using the GraphPad Prism software package, version 3.0 (GraphPad Prism). All experiments were conducted at least three times. The number of independent experiments is indicated by n. Significance was considered at p < 0.05.
Results

Generation of lacrimal gland organoids from human tissues

To generate lacrimal gland organoids, we used human lacrimal gland tissues and allowed them to self-organize within Matrigel(Fig.1A). Dissociated lacrimal gland cells were embedded in Matrigel and then grown respectively in five media conditionsby modifying human prostate or salivary gland organoid media. Most organoids were generated under all medium conditions. In the M-SA1 medium (salivary gland organoid media containing 10 mM Nicotinamide, 500 nM A83, and 100 ng/ml Noggin) group, however, the formation and growth of organoid was maintained following passage, unlike other groups. Therefore, we defined M-SA1 medium as a lacrimal gland organoid medium (LGOM) (Fig.1B). In the LGOM, lacrimal gland organoids were expanded until passage 19 (Fig. 1B) and increased in size until day 15 (Fig. 1C).Histological analysis showed that organoids were similar to lacrimal gland tissues. Acinar cells as the major cell type of lacrimal gland tissue and its secreted acidic mucosubstances were observed (Fig.1D, H&E, and Alcianblue staining, respectively). Secretory products such as glycogen and glycoprotein were also observed in normal lacrimal tissue and organoids (Fig. 1D, PAS staining). Masson's trichrome staining showed that keratin was the major ECM component of organoids (Fig. 1D). These results showed that organoids developed in this study have similar morphology with acinar cells of lacrimal gland tissue.

Additionally, we confirmed the expression of EpCAM (epithelial cell adhesion molecule), an epithelial lineage cells marker, in the lacrimal tissue (Fig. 1E).EpCAM-positive cells generated more lacrimal gland organoids than a non-sorted fraction, whereas organoids were not formed in EpCAM-negative cell fraction (Fig. 1F). A larger amount of vimentin, a gland specific marker, was observed in organoids from EpCAM-positive cells. These results showed that the origin of organoid formation was EpCAM-positive cell(Fig. 1G).

Human lacrimal gland organoids recapitulate structural properties of human tissues

Developed lacrimal gland organoids were compared with human lacrimal gland tissues. Specific markers such as Aquaporin-5 (AQP5), Lysozyme (LYZ), E-cadherin(E-CAD), Vimentin (VIM), andα-SMA were used for immunofluorescence staining (Fig. 2A). Expression patterns of Vimentin, AQP5, and LYZ in organoids were similar to those in acinar cells of lacrimal gland tissues. Also, expression pattern of E-CAD and α-SMA in lacrimal gland organoids shows a similar to those in tissue, although their expression levels were lower than those in lacrimal tissues. These markers are known to express in acinar cells, but not in ductal cells. Therefore, formed organoids in this study are more similar to acinar cells than to ductal cells in lacrimal tissues. Additionally, we confirmed proliferating cells in the lacrimal gland organoid by BrdU assay (Fig. 2B). At 4 days after BrdU treatment, BrdU positive cells showed Ki67 in the outer region of organoids, unlike that in the inner region of organoids. Especially, vimentin expression was observed around BrdU-positive cells.
To determine cellular ultrastructure, lacrimal gland organoids were analyzed by TEM. In some organoids, cells (Fig. 2C, white dotted line) having a secretory granule (Fig. 2C, black arrowhead) were observed in organoids. These characteristics are known to be features of acinar cells.

**Human lacrimal gland organoids recapitulate structural properties of human lacrimal glands**

In this study, we confirmed the secretory function of developed organoids by pilocarpine stimulation. After stimulation with pilocarpine (1μg/ml), the concentration of calcium ions was increased in cells of organoids (Fig. 3A). In cells of the lacrimal gland, increasing concentration of calcium ion is known to lead to tear secretion [29]. Secretion of β-Hexosaminidase, a lysosomal enzyme, was also increased in pilocarpine treated organoids (Fig. 3B). Especially, secreted proteins were observed (Fig. 3C, red arrowhead) and cell-cell junction was widened (Fig. 3C, asterisk) in pilocarpine treated organoids. These results indicate that organoids developed in the present study have a secretory function in response to cholinergic agonist pilocarpine, similar to lacrimal gland tissue.

**Human lacrimal gland organoids recapitulate functional properties of human lacrimal glands**

To identify secreted proteins by pilocarpine in lacrimal gland organoids, the culture medium was collected and analyzed for proteomics after 2 hours of PBS or pilocarpine treatment. A total of 776 proteins were identified and quantified in both groups, the amount of the proteins was additionally secreted by pilocarpine (Fig. 3D-E). In the pilocarpine treated organoid, up-regulated cellular components were found to be extracellular exosome/vehicles by Gene Ontology (GO) analysis (Fig. 3E-F). Additionally, we identified a total of 66 differentially expressed proteins (DEPs, fold change > 2) and most proteins (more than 70%) are belong to exosome/vehicle (Fig. 3G). Among the upregulated proteins in the pilocarpine treated group, the protein synthesis-related secretome (such as RPS, RPL family, etc.) was noticeably increased. These results mean that our developed lacrimal organoids might turn on the synthetic process such as tear production by the stimulator.

**Generation of lacrimal gland organoids derived from lacrimal glands of Sjogren's syndrome (SS) patients**

To advance our research, we developed lacrimal gland organoids from the tissue of patients with Sjogren's syndrome (SS); these are referred to as SS organoids. SS organoids were generated and cultured following the same method used for normal organoids. SS organoids featured a smaller size and were cultured for shorter passages (up to passage 2), as compared to normal organoids (Fig. 4A). Expression of AQP5, the lacrimal gland specific marker, was markedly decreased in the SS tissue compared to the normal tissue (Fig. 4B). Similarly, the expression of those markers was decreased in the SS organoids as compared with the normal organoids (Fig. 4B and C). Besides, histological analysis revealed that SS organoids have higher fibroblastic properties than those comprised of normal tissue and organoids, while their degree is similar to the SS tissue (Fig. 4D). Morphologically, some structural damages in acinar cells were found in the H&E analysis and dense blue regions representing collagen-stained areas in the Masson's trichrome staining of SS organoids. This is matched with those of the SS tissue, whereas the normal organoid shows an intact structure in the light blue area as in the normal
tissue. These results suggest that our developed SS organoids could be a useful tool in vitro model mimetic SS disease.

**Engraftment of lacrimal gland organoids in the mouse model of dry eye syndrome**

To confirm the engraftment ability of lacrimal gland organoids, the organoids from the lacrimal gland tissue of C57BL/6-GFP-Tg mice were prepared following the method used for human organoids. GFP-labeled organoids were formed (Fig. 5A, a–b), and their morphology was similar to acinar cells of the mouse lacrimal tissue (Fig. 5A, c–d). In this study, we established the inflammation-based dry eye disease (DED) mouse model by administering a ConA injection into the extra-orbital gland of the lacrimal tissue and transplanting the formed GFP-organoids into the lacrimal tissue of the DED mouse. GFP signals were observed in the mouse lacrimal tissue after 14 days of transplantation (Fig. 5B). Additionally, we confirmed that AQP5, known as water channel protein for tear production, was expressed with transplanted organoid (Fig 5B). These results suggest the therapeutic potential of lacrimal gland organoids for the regeneration of the DED due to the injured lacrimal tissue.

**Discussion**

The lacrimal gland plays an essential role in lubricating the ocular surface epithelium, similar to other exocrine glands of the body [4]. Both lacrimal and salivary gland acinar cells share developmental, morphologic, and functional characteristics. They also share similar reduction in function when injured [31-32]. A preliminary report has shown that stem cells in mouse lacrimal gland can be subsequently cultured *in vitro* [21]. There was an evidence showing stem/progenitor cell compartment in the terminal ductule in the salivary gland [14]. This result proves that human lacrimal gland cells could be cultured *in vitro* and retain their secretory function. In addition to the presence of differentiated cells (epithelial, myoepithelial, and stromal), we could recapitulate the real gland as an ‘organoid’ with typical cell to cell junctional connections.

Previously simulated microgravity has promoted the development of spheroidal aggregates of rabbit lacrimal gland cells with a mean diameter of 384.6±111.8µm after 7 days [24]. These spheroids consisted of organized lacrimal gland cells and acini-like structures. The mean diameter of these spheroids was maintained continuously over a culture period of about 28 days. However, apoptosis was found at the center of spheroid aggregates, correlating with diameter of spheroids and duration of the culture period (between days 14 and 21 and between days 21 and 28) [24]. Our culture condition could provide a stable environment in terms of the size and integrity of lacrimal organoid growth from human lacrimal gland stem cells.

Another method to differentiate iPSCs towards a lacrimal gland epithelial cell phenotype would require development process, although this has already been described forembryonic stem cells [25]. Spheroids can be formed by assembling three cell types: epithelial cells, MSCs isolated from porcine lacrimal gland, and endothelial cells isolated from human foreskin [33]. They retained their own immunophenotypes with spontaneous organization in response to parasympathetic...
stimulation, although spheroid function, cell proliferation, and viability decreased with apoptosis over time [33]. This study demonstrated the possibility of constructing a true lacrimal gland functional unit, comprising lacrimal epithelial cells and MSCs from adult mammalian tissues in vitro.

Much effort has been put into the culture of lacrimal gland in vitro. For example, experiments in a micrograft environment and transfer of amniotic membranes have been performed. However, these methods have not shown expected results over an extended duration [34]. We explored five media conditions by modifying the salivary gland organoid media and applied them to organoid culture. We finally concluded that M-Sa1 medium would be the best lacrimal gland organoid medium because it resulted in the longest surface, the maximum length of organoids formed, and the best expansion. Similarly, murine lacrimal gland stem cells can express markers of stemness (such as Nanog, Sox2, and Klf4) known as early-lineage markers of all three germ layers [34]. In the present study, lacrimal gland organoids from normal person presented specific gland markers such as vimentin, E-cad, Aqp5, and α-SMA, although their expression was lower than that of tissue. As shown in Fig4, also, the expression pattern of specific markers between organoids from normal and SS patients reflects the difference between the actual two tissues. Sjogren patients who produce less tear secretion than a normal person, have an inability in the Ca2+-signaling pathway. Sjogren patients have no Ca2+ channel that exists in the myoepithelial cell plasma membrane [Garcia-Posadas, 2020 #96]. Myoepithelial cell altered in Sjogren patients with α-SMA than the normal person. These results suggest that our developed SS organoids could be a useful tool in vitro model mimetic SS disease.

In this study, we tried to confirm the secretory function of the lacrimal gland organoid and measured the secretory function in vitro. Muscarinic receptors should be activated by various stimulants to induce tear secretion from the lacrimal gland. Under the condition, internal Ca2+ ions increase, and it leads to tear secretion [Imada, 2017 #97]. Pilocarpine is a parasympathetic stimulant mainly with a stimulatory effect on muscarinic M3 receptors. Since organoids do not have nerves, they are to be stimulated using pilocarpine, an M3 receptor agonist. As a result, internal Ca2+ions and β-hexosaminidase as known a lysosomal protein in tear fluid was increased in the pilocarpine treated organoid. Also, the secreted proteins from pilocarpine treated lacrimal organoid were identified through proteomics. These results indicate that our developed organoid has a reactivity to pilocarpine, which shows the function of the lacrimal gland.

This study also demonstrated preliminary evidence that the lacrimal gland organoid contained a self-repair effect of stem cells in an inflammation-induced dry eye animal model. However, its future role merits further investigation. Although dry eye syndrome has a moderate prevalence globally, the mainstay of clinical management is still conservative using artificial tear drops and lubricants [4]. Transplanted MSCs are well known to enhance corneal wound healing by trophic factor production and immune regulatory effect rather than by direct transdifferentiation into epithelial cells [35-37]. As pre-requisites for cell therapy, the plausibility of using lacrimal organoids containing functional stem cells to rescue and repair to obtain functionally competent cells should be studied. Such studies could contribute to regeneration of damaged tissues [23].
We tried to determine the possibility of regenerating damaged lacrimal gland using lacrimal organoids after inducing inflammation using concanavalin A in C57BL / 6 mice. Such damage to lacrimal gland owing to aging, hormonal imbalance, or radiation could be treated using organoid based on this study. Thus, organoid is applicable in the future. In summary, this study provided the first evidence for successful growth of fresh human lacrimal gland organoid *in vitro* with an attempt toward functional unit formation while retaining secretory function. Further validation of findings of this study is needed to allow the development of a functionally competent secretory lacrimal organoid for potential clinical application in severe cases of dry eyes including autoimmune lacrimal gland disorders.

**Conclusion**

In conclusion, we established lacrimal organoids from human and mouse lacrimal tissue. The organoids established in this study recapitulate the structure and function of human lacrimal glands. Organoids can be used as a tool that can be used in disease modeling such as Sjogren's syndrome and further in the field of regenerative therapy.

**Declarations**

**Authors' contributions**

J.S.Y.&C.W.H.; Collection and/or assembly of data, manuscript writing: J.S.G.; Collection and/or assembly of data, manuscript writing for revision: L.S.; Assembly of data: P.J.M.&L.H.; Proteomics analysis: P.M.; Assembly of data: L.H.; Conception and design/Manuscript writing: Y.J.; Conception and design/Manuscript writing/Final approval of the manuscript.

**Authors’ information**

Sang Yun Jeong and Woo Hee Choi contributed equally to this work.

**Funding**

This work was supported by the Basic Science Research Program through the National Research Foundation of Korea funded by the Ministry of Science, ICT and Future Planning, Republic of Korea (NRF-2018R1D1A102050030), by a grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute, funded by the Ministry of Health & Welfare (HI16C0002 and HI18C2458), and by the Technology Innovation Program (or Industrial Strategic Technology Development Program-3D-TissueChip Based Drug Discovery Platform Program) (20009773, Commercialization of 3D Multifunction Tissue Mimetics Based Drug Evaluation Platform) funded by the Ministry of Trade, Industry & Energy (MOTIE, Korea).

**Availability of data and materials**
The datasets generated during and/or analyzed during the study are available from the corresponding author on reasonable request.

**Ethical Approval and Consent to participate**

This study was approved by the institutional review board (IRB) of CHA Bundang medical center, CHA University (CHAMC 2018-01-007) and carried out with the written consent of all donors.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declared no potential conflicts of interest.

**Acknowledgements**

Not applicable.

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**Abbreviations**

DED: Dry eye disease; LGOM: lacrimal gland organoid medium; PR: medium for prostate organoid cultivation; SA: medium for salivary organoid cultivation; M-SA1/M-SA2: modified from salivary organoid culture medium; M-PRSA: modified from prostate and salivary organoid culture medium; VIM: vimentin; E-CAD: E-cadherin; AQP5: aquaporin5; α-SMA: alpha-smooth muscle actin; LYZ: lysozyme; SS: Sjogren’s syndrome; ConA: Concanavalin A

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Figures
Functional recapitualation of human lacrimal gland organoids. To confirm the secretory function of organoid, (A) Ca2+ uptake (Magnification; 10X, Scale bar; 100 um) and (B) released β–hexosaminidase was detected after pilocarpine treatment (1 ug/ml) and (C) the secretome released from the organoid was observed by TEM analysis. The secreted proteins by pilocarpine were identified using Proteomic analysis. Through the (D) Heatmap, (E) Venn diagram, (F) GO (Gene Ontology) term analysis and identification of the DEPs (differentially expressed proteins), proteins were more secreted from pilocarpine treated organoids and most proteins belong to exosome/vehicle.