Evaluation of Decellularized Porcine Jejunum as a Matrix for Lacrimal Gland Reconstruction In Vitro for Treatment of Dry Eye Syndrome

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Submitted: September 16, 2016
Accepted: September 4, 2017

PURPOSE. Dry eye syndrome (DES) can cause blindness in severe cases, but mainly palliative treatments exist. A tissue-engineered lacrimal gland (LG) could provide a curative treatment. We aimed to evaluate decellularized porcine jejunum (SIS-Muc) as a scaffold for porcine LG epithelial cells.

METHODS. To evaluate SIS-Muc as a potential scaffold, basement membrane proteins in SIS-Muc and native LG were compared (immunohistochemistry [IHC]). Porcine LG epithelial cells cultured on plastic were characterized (immunocytochemistry), and their culture supernatant was compared with porcine tears (proteomics). Epithelial cells were then seeded onto SIS-Muc in either a static (cell crown) or dynamic culture (within a perfusion chamber) and metabolic (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) and secretory capacities (β-hexosaminidase assay), protein expression (IHC), and ultrastructure transmission electron microscopy (TEM) compared in each.

RESULTS. Collagen IV and laminin were found in both native LG and SIS-Muc. When cultured on plastic, LG epithelial cells expressed pan-cytokeratin, Rab3D, HexA, and produced mucins, but lysozyme and lactoferrin expression was nearly absent. Some porcine tear proteins (lipocalin-2 and lactoferin) were found in LG epithelial cell culture supernatants. When LG cells were cultured on SIS-Muc, metabolic and β-hexosaminidase activities were greater in dynamic cultures than static cultures (P < 0.05). In both static and dynamic cultures, cells expressed pan-cytokeratin, Rab3D, lysozyme, and lactoferin and produced mucins, and TEM revealed cell polarization at the apical surface and cell–cell and cell–scaffold contacts.

CONCLUSIONS. SIS-Muc is a suitable scaffold for LG cell expansion and may be useful toward reconstruction of LG tissue to provide a curative treatment for DES. Dynamic culture enhances cell metabolic and functional activities.

Keywords: lacrimal gland, dry eye syndrome, tissue engineering, regenerative medicine, decellularized scaffold

Dry eye syndrome (DES) is a moderately prevalent, complex, multifactorial disease that, in severe cases, can result in loss of vision.1 Despite this, mainly palliative treatment options exist, which aim either to replace lost tear volume or to reduce inflammation.2 Development of a curative treatment would be beneficial. As such, auto-transplantation of salivary gland tissue has been proposed, but because the composition of saliva differs from lacrimal fluid, this can result in further damage to ocular surface epithelium.3–5 Xenotransplantation of porcine lacrimal glands (LGs) has also been considered but this would require lifelong immunosuppression and thus far serious risks such as thrombocytopenia, complement-induced graft injury, and possible differences between animal and human physiology interdict testing of xenotransplantation in clinical trials.5,6 and there is a risk of porcine endogenous retrovirus transmission.8 A regenerative medicine treatment approach to provide a curative treatment for DES is therefore desirable. A major cause of severe DES is dysfunction of the LG, which results in production of lacrimal fluid in either insufficient volume, or of abnormal composition, or both. Lacrimal fluid is produced by LG epithelial cells and so the normal function of these cells is crucial to maintenance of the tear film.2,9,10 It follows that this cell type should form the basis of any LG reconstructed in vitro. Techniques for isolation and culture of these cells are now established and numerous scaffolds, both natural and synthetic, have been evaluated for their suitability to support their growth.11–17 Approaches to permit three-dimensional (3D) culture of LG epithelial cells have also been
investigated, and such formats may help to promote cell function. 3D scaffolds are also useful since they permit efficient transplantation (i.e., over injection of single cell suspensions) of large cell numbers.

Decellularized tissues are an attractive scaffold option because their application does not trigger an immune response, they are inherently biomimetic, with appropriate mechanical properties and protein composition. Decellularized LG tissue in particular would also provide tissue-specific architecture and decellularized rabbit and porcine LG have been evaluated as 3D scaffolds for LG culture and transplantation and do appear promising. However, such tissue is technically challenging to harvest and not readily available. Conversely decellularized porcine small intestine submucosa (SIS-Muc) may readily be harvested and has already been shown to support growth of numerous cell types including cells derived from nerve sheath, lung and colorectal epithelia, tumors, and also primary hepatocytes and bronchial epithelia cells. Our first aim was, therefore, to evaluate SIS-Muc as a scaffold for LG epithelial cells.

Second, we aimed to establish whether dynamic culture might be beneficial for epithelial cell cultures on SIS-Muc. Dynamic cultures are more physiologic than static and have been shown to promote proliferation and function in other cell types. Dynamic culture of LG epithelial cells has also been performed previously using a rotating cell culture system and appeared promising. However, in that system, the cells were cultured without a support of a scaffold, which differs from what is proposed here. Furthermore, no direct comparison was made between static and dynamic culture (in the rotating cell culture system) meaning that the benefit of dynamic culture for LG epithelial cells has not yet been proven.

Overall, we aimed to investigate the feasibility of LG epithelial cell culture on decellularised porcine jejunum (SIS-Muc) under dynamic culture for in vitro reconstruction of LG tissue for treatment of DES.

METHODS

Materials

All materials were from Sigma-Aldrich (Schnelldorf, Germany) unless otherwise stated. All antibodies were from Abcam (Cambridge, UK) unless otherwise stated.

Harvesting of Porcine LGs

LGs were harvested from 8-week-old pigs (Sus scrofa) and transported to the laboratory within 1 hour postmortem.

IHC of Native LG and SIS-Muc

SIS-Muc was processed as described previously and obtained from the Department of Tissue Engineering and Regenerative Medicine, University Hospital Würzburg, Würzburg. Briefly, intact tissue was decellularized using cycles of sodium deoxycholate and DNase.

Samples were fixed in 4% paraformaldehyde (PFA) before processing and embedding using standard protocols. Four-micrometer sections were cut, dewaxed, and rehydrated. Antigen retrieval was performed using 10 mM sodium citrate buffer (pH 6) at 95°C for 20 minutes. Donkey serum (5%) was added to block non-specific binding sites.

For antigen retrieval, samples were incubated in 2% donkey serum at 4°C for 20 minutes. Donkey serum (5%) was added to block non-specific binding sites. Samples were then incubated with appropriate antibodies overnight at 4°C. After washing, appropriate detection antibodies were applied. Detection antibodies were applied as follows:

- Rab3D (ab170057) (1:200)
- Lysozyme (ab74666) (undiluted)
- Pan-cytokeratin (53-9003-82, eBioscience, Frankfurt, Germany) (1:500)
- Rab3D (ab170057) (1:200)

Culture of LG Epithelial Cells From LG Explants

LG tissue was cut into pieces measuring approximately 1 mm3, and these were used to initiate explant cultures of epithelial cells. Cells were cultured in DMEM:F12 supplemented with 10% FBS, 0.4 µg/mL hydrocortisone, 0.1 nM cholera toxin, 0.075% sodium bicarbonate, 0.18 mM adenine, 2 nM T3, 3 µg/µL transferrin, 5 µg/µL insulin, 1X antibiotic, antimycotic, and 10 ng/mL epidermal growth factor (EGF). Medium was changed three times per week, and cells were maintained in a humidified incubator at 37°C, 5% CO2. After approximately 2 weeks, epithelial cells were trypsinized and used for either immunocytochemistry, secretome analysis, or seeded onto SIS-Muc (i.e., at first passage).

Immunocytochemistry

First passage epithelial cells were seeded into eight-well chamber slides, left to adhere overnight and fixed using 4% PFA; 0.5% bovine serum albumin (BSA; wt/vol) in PBS containing 0.1% Triton X-100 was used for blocking for 1 hour. Capture antibodies were applied overnight at 4°C in 0.5% BSA (wt/vol) in PBS as follows: z-smooth muscle actin (aSMA) (Dako, M0851) (1:100), CK7 (1:50), CK19 (1:50), HexA (NB01-7147; NovusBio, Abingdon, UK) (1:100); lactoferrin (141200; US Biological, Salem, MA, USA) (1:200); lysozyme (ab74666) (undiluted); pan-cytokeratin (53-9003-82, ebioScience, Frankfurt, Germany) (1:500); and Rab3D (ab170057) (1:200). The next day, detection antibodies were applied, and samples were mounted as previously described.

Periodic Acid Schiff Alcian Blue Staining

For periodic acid Schiff (PAS)-Alcian blue staining, first passage cells were stained with Alcian blue for 4 minutes and PAS reagent for 15 minutes.

Secretome Analysis

Tears were collected from three pigs as described previously. Briefly, filter paper was used to blot tears before centrifuging to collect tears. All experimental methods and animal care procedures adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. For analysis of epithelial cell culture supernatants, 0.5 × 106 first passage cells were seeded into 24-well plates. On day 3, cells were washed with serum-free DMEM and incubated in 100 µl carbachol for 30 minutes under normal culture conditions. The conditioned media samples were collected and stored at –80°C until analysis using shotgun mass spectrometry.

Protein samples were shorty stacked in a 4% to 12% polyacrylamide bis-tris gel (+mm running distance) (Life Technologies, Darmstadt, Germany), and after silver staining, protein containing bands were cut out, destained, reduced, alkylated, tryptic digested, and prepared for mass spectrometry as described previously. Peptides were separated by a 2-hour gradient using an Ultimate 3000 Rapid Separation liquid chromatography system ( Dionex/Thermo Scientific, Idstein, Germany) and analyzed on an Orbitrap Elite high-resolution instrument (Thermo Scientific, Bremen, Germany) as previously described.
For protein and peptide identification and further processing, Proteome Discoverer (version 1.4.1.14; Thermo Scientific, Dreieich, Germany) connected to a Mascot server (version 2.4.1; Matrix Sciences, London, UK) was used. Spectra from the analysis were searched against Sus scrofa reference proteome entries from UniProtKB (26,143 entries, downloaded March 9, 2014). The following search parameters were applied: mass tolerance precursor, 10 ppm; mass tolerance fragment spectra, 0.4 Da; trypsin-specific cleavage with a maximum of one missed cleavage site; carbamidomethyl as fixed modification; and methionine oxidation and N-terminal acetylation as variable modifications. For peptide and protein acceptance, the Percolator was used with a strict target false discovery rate (FDR) of 0.01. Protein grouping was enabled, and proteins identified with a minimum of two peptides were reported. The number of peptide spectrum matches was used as semiquantitative correlate for the relative amount of proteins to compare different samples.

**Culture of Epithelial Cells on SIS-Muc**

The SIS-Muc was cut and assembled into the cell crowns (Fig. 1, static culture) so that the former lumen faced upward. SIS-Muc was left overnight in medium. Then, $2 \times 10^5$ first passage cells were seeded onto the former lumen (mucosa). Cells were maintained at 37°C in a 5% CO$_2$ humidified incubator with daily medium changes.

For dynamic culture, after 2 days of static culture, SIS-Muc was carefully transferred to a perfusion chamber to permit continuous flow of medium over the surface of the mucosa seeded with epithelial cells. The whole circuit was assembled as in Figure 1 and placed into a bioreactor to maintain normal culture conditions. The peristaltic pump was started to fill the circuit (at a flow rate of 2.5 mL/min). The next day, the flow rate was increased to 3.8 mL/min and cultured for a further 6 days.

**Measurement of Metabolic Activity of Recellularized SIS-Muc**

Metabolic activity was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Samples were incubated with MTT (0.5 mg/mL) for 30 minutes under normal culture conditions. Formazan crystals were dissolved using isopropanol. Optical density (570 nm) of samples and blanks (SIS-Muc without cells) was measured using a plate reader (Multiskan EX; Thermo Scientific).

**Histology and IHC of Recellularized SIS-Muc**

Four-micrometer sections were stained with hematoxylin for 5 minutes and eosin for 7 minutes (H&E) or with PAS-Alcian blue for 7 minutes. This medium was then collected and centrifuged to remove cellular debris. After fixation, samples were incubated in 1% osmium tetroxide in 0.1 M cacodylate buffer for 2 hours. Samples were dehydrated using acetone (50%, 70%, 90%, and 100%), and contrast agent was added (1% phosphotungstic acid/0.5% uranylacetate in 70% acetone). Samples were embedded using a SPURR embedding kit (Serva, Heidelberg, Germany), polymerized overnight at 70°C, and cut into 80-nm sections using an Ultracut EM UC7 (Leica). An H600 TEM (Hitachi, Tokyo, Japan) was used to capture the images at 75 kV. At least 10 images were captured per condition.

**Quantification of Secretory Capacity–β-Hexosaminidase Activity Assay**

Cells on SIS-Muc were washed three times in serum-free medium and incubated for 2 hours in 500 μL serum-free medium under normal culture conditions, before 320 μL of this was collected and centrifuged to remove cellular debris (baseline sample). Then, 20 μL 1 mM carbachol was added to the remaining 180 μL of medium (final carbachol concentration was 100 μM), and cells were incubated for a further 30 minutes. This medium was then collected and centrifuged (carbachol sample). Cell-free samples were also included that were collected identically. All samples were stored at –80°C until analysis, which was performed as described previously. Briefly, 20 μL sample was incubated with 20 μL water and 50 μL 7.5 mM 4-methylumbelliferyl-N-acetyl-β-D-glucosaminidase

**Static**

**Dynamic**

![Figure 1. Setup for static and dynamic cultures of LG epithelial cells on SIS-Muc.](insert)

For static cultures, self-constructed cell crowns (top left) were used. SIS-Muc was clamped between the two portions and cultured in 12-well plates (top right). SIS-Muc position is indicated (red dashed line). For dynamic cultures, SIS-Muc was clamped in place within self-constructed perfusion chambers (3, bottom). Culture medium was pumped from the medium reservoir (1) across the SIS-Muc within the perfusion chambers using a pump (2). The entire circuit was maintained at 37°C, 5% CO$_2$ within a bioreactor.
(substrate) for 2 hours in the dark. Fluorescence (365/460) was measured using a plate reader.

Quantification of Secretory Capacity–Lactoferrin ELISA

On day 9 of culture, samples were washed with serum-free DMEM and incubated with DMEM for 24 hours under normal culture conditions. Conditioned medium samples were stored at -80°C until analysis. An indirect ELISA was used to quantify lactoferrin concentration. Fifty microliters of sample was pipetted into individual wells of a 96-well immunoplate and incubated overnight at 4°C. The next day, the plate was washed with PBS three times before blocking buffer (5% milk powder in PBS, with 0.05% Tween 20) was applied for 90 minutes. An anti-porcine lactoferrin capture antibody (VWR, 141200; 1:200) was added and left overnight at 4°C. The next day, the plate was washed again with PBS three times, before an anti-rabbit IgG horseradish peroxidase (HRP)-linked detection antibody (ab6802) (1:10,000) was applied for 90 minutes. o-phenylenediamine dihydrochloride (OPD) was used for detection, and the reaction was stopped using 2 M sulphuric acid. Optical density (492 nm) was measured using a plate reader. Recombinant porcine lactoferrin (RPA780Po01; USCN, Houston, TX, USA) was used to generate a standard curve (100 to 1.5625 ng/mL) for quantification.

RESULTS

SIS-Muc Retains Basement Membrane Profile

IHC of native LG revealed that acini structures were surrounded by laminin and collagen IV (Fig. 2). IHC of SIS-Muc revealed no positive DAPI staining, confirming that porcine cellular material had been removed. Laminin and collagen IV basement membrane proteins were still present after decellularization and were found on the apical surface of the submucosa, lining blood vessels, and at the submucosa–mucosa interface.

Porcine LG Epithelial Cell Characterization

Heat mapping (Fig. 3A) revealed that a number of tear film proteins, including lipocalin-2, complement C3, serotransferrin, lactoferrin, serum albumin, mucin-4, and α-2-macroglobulin, were present in both pig tears and culture supernatant (albeit it at lower levels). However, mucin-13, lysozyme C-1, -2, and -3, and lipocalin-1 were only detected in tears. HexA was also detectable in pig culture supernatants (data not shown). Secretome analysis revealed that a total of 769 proteins were detected in pig tear samples, and 386 of these proteins were also detected in pig culture supernatants (Fig. 3B).

In native LG, CK19 expression was found in both acini and duct regions, whereas CK7 expression was limited exclusively to ducts, and aSMA expression was observed around individual acini (Fig. 3C). Cultured LG epithelial cells displayed typical epithelial cell morphology (Fig. 3D) and almost all expressed CK19, whereas only a few cells expressed CK7 or aSMA. Cells also stained positively for both PAS and Alcian blue, indicating continued secretion of both acidic and neutral mucins. Immunocytochemistry revealed that almost all cells expressed pan-cytokeratin (confirming epithelial origin) and Rab5D and HexA (confirming secretory capacity), whereas only very few expressed lysozyme or lactoferrin (indicating secretion of LG-specific proteins).
Figure 3. LG epithelial cell characterization. (A) Heat map showing relative abundance of selected proteins in porcine tear and culture supernatants. Red to yellow, high to low measured intensity; gray, undetected. (B) Venn diagram summarizing secretome analysis, showing proteins detected in porcine tears and in porcine LG epithelial culture supernatant. (C) Immunohistochemical staining of native LG against CK19 and aSMA (red) and CK7 (green) with DAPI nuclear counterstain (blue). (D) (Immunocytochemical staining of epithelial cells on plastic: PAS Alcian blue, CK19 (red), CK7 (green), pan-cytokeratin (green); aSMA; Rab3D; HexA; lysozyme and lactoferrin (all red) with DAPI nuclear counterstain (blue). Scale bar denotes 100 μm (applies to all images).
SIS-Muc Supports LG Epithelial Cell Growth and Dynamic Culture Promotes Metabolic and Functional Activities

Histology of LG epithelial cells on SIS-Muc on day 2 revealed a monolayer present on the surface of the mucosa. In day 6 static cultures, epithelial cells had filled mucosal crypts. Day 9 static cultures appeared similar to day 6 static cultures. Conversely, in day 6 dynamic cultures, epithelial cells had not only filled mucosal crypts but were evident throughout the mucosa. By day 9 of dynamic culture, epithelial cells appeared to have completely remodeled the mucosa to form a cell layer up to eight cells deep (Fig. 4A). We did not see any evidence of cell migration into the submucosa.

On day 9, qualitative evaluation of metabolic activity using MTT revealed confluency over the entire SIS-Muc surface, with stronger staining intensity in dynamic cultures compared with static. Quantitative measurement showed that the metabolic activity in dynamic cultures was more than twice that of static cultures ($P < 0.05$; Fig. 4B).

Ultrastructure of LG Epithelial Cells on SIS-Muc Is Similar to That in Native Tissue

TEM analysis of cells cultured on SIS-Muc revealed some evidence of cell polarization at the superior surface: apical microvilli and basal cell nuclei were apparent in both static and dynamic culture formats (Fig. 5). In both culture conditions, cells were present that contained secretory vesicles of differing electron densities that appeared to be directed toward the apical surfaces. Cell polarization was also observed within mucosal crypts in static cultures, where secretory vesicles were localized to one end of the cell. In dynamic cultures only, numerous multilamellar bodies were also evident. In neither static nor dynamic cultures, however, was there any evidence of organization into acini-like structures even within mucosal crypts. Cells were well adhered to SIS-Muc in both conditions with numerous interdigitations. LG epithelial cells in both static and dynamic cultures had formed cell–cell contacts such as desmosomes and tight junctions.

LG Epithelial Cell Immunophenotype Is Maintained on SIS-Muc

Qualitative assessment of the LG epithelial cell phenotype on SIS-Muc using IHC on day 9 revealed that almost all cells in both static and dynamic cultures stained positively for pan-cytokeratin (confirming epithelial origin), PAS-Alcian blue (indicating continued mucin secretion), and Rab3D (indicating presence of secretory vesicles), whereas only a few expressed aSMA (Fig. 6). Similarly, many (although not all) cells expressed lactoferrin and lysozyme, confirming production of proteins present in tear film. There appeared to be a trend toward increased lactoferrin and lysozyme expression within superior epithelial cells in dynamic cultures.

Secretory Capacity of LG Epithelial Cells on SIS-Muc Is Upregulated in Dynamic Cultures Compared With Static Cultures

The $\beta$-hexosaminidase assay revealed a statistically significant response to carbachol on day 2 ($P < 0.05$; Fig. 7). On day 9, there was also a tendency to respond to carbachol for both static and dynamic cultures, but this was not statistically significant. Epithelial cells that had been maintained in static culture showed only a small increase in overall secretory
activity between days 2 and 9. Conversely, those that had been maintained in dynamic culture showed a far larger increase in activity over day 2 (approximately sixfold). Importantly, secretory activity was increased three- to fourfold in epithelial cells that had been cultured in dynamic culture compared with static-cultured cells on day 9 ($P < 0.05$ without carbachol; $P < 0.01$ with carbachol). Expression of the M3 muscarinic receptor appeared to increase slightly between days 2 and 9.

**FIGURE 5.** Ultrastructural characterization of epithelial cells on SIS-Muc. Epithelial cells on SIS-Muc were examined using TEM. **Top:** at the apical surface in both static and dynamic cultures, microvilli (mv), secretory vesicles (pink boxes), and basal nuclei (n) were evident. In dynamic cultures, multilamellar bodies (green arrowheads) were also observed. In mucosal crypts, cells were not orientated against the SIS-Muc. **Middle:** Epithelial cells were well adhered to collagenous SIS-Muc in both static and dynamic cultures. In mucosal crypts, cell polarization was apparent with secretory vesicles (pink boxes) apparent. **Bottom:** Epithelial cells had formed cell–cell contacts such as tight junctions (black arrowheads) and desmosomes (white arrowheads). Images are of one biological repeat per condition.
Lactoferrin in culture supernatant was undetectable in ELISA for both static and dynamic cultures.

**DISCUSSION**

DES is a complex, moderately prevalent disease that in severe cases can cause blindness, for which no curative treatment exists. In the current study, we aimed to evaluate SIS-Muc as a scaffold for LG epithelial cells. As for other decellularized scaffolds, we found that SIS-Muc is inherently biomimetic and retains a basement protein profile (collagen IV and laminin) similar to those of native tissue. Because environmental cues (such as cell–matrix interactions) are known to impact on cell phenotype, this is highly relevant when attempting to reconstruct tissue in vitro. An additional advantage of SIS-Muc is that it may be harvested with intact vasculature that may be reseeded with endothelial cells to restore functionality; a major benefit as the number of cells that may be supported by SIS-Muc will be increased, potentially permitting scale-up to clinically useful dimensions.

In our study, we used LG epithelial cells derived from porcine explants. Ideally, human cells would be used for such evaluations, but human LG tissue is very rarely available. The porcine LG has shown to be very similar to the human LG in terms of its macro- and micro-anatomy, as well as its blood supply. Here we demonstrated using proteomics that porcine tears contain important tear film proteins such as lysozyme,
lipocalin, transferrin, lactoferrin, and albumin, which are present in human tears and required for maintenance of a normal tear film.\textsuperscript{36} We were, however, unable to detect some common tear proteins such as lysozymes and lipocalin-1 in culture supernatants, and secretion of others (such as lactoferrin) was found at lower levels than in tears. This may be due to the short incubation period used to obtain the samples (2.5 hours) or, more likely, that the cells were poorly differentiated at this stage of culture. Cultured LG cells are known to be highly proliferative,\textsuperscript{22} and here by immunocytochemistry we found only very few cells expressing lysozyme and lactoferrin, whereas expression of general secretory markers, such as HexA and Rab3D, was widespread. This is not necessarily problematic providing that these cells are capable of differentiation into “functional” LG epithelial cells. Indeed, application of poorly differentiated cells may produce a more long-lived graft in vivo because regenerative capacity may be better retained. Similar arguments have been made for reconstruction of other ocular surface tissues such as cornea, where poorly differentiated limbal epithelial stem cells (known to be capable of differentiation into corneal epithelial cells) are transplanted.\textsuperscript{37} Comparison of expression of CK7, CK19, and αSMA in native tissue with cultured cells also demonstrated that the majority of our cultured cells were acinar cells, although some ductal cells and myoepithelial were present. All three cell types are required for complete function of LG: to secrete and expel tear film proteins and to modify the composition of the tear film thereafter if required, and therefore having a mixed population is advantageous.

When LG epithelial cells were cultured on SIS-Muc in static cultures, cells filled mucosal crypts and formed a cell layer on the superior surface, indicating that SIS-Muc could support LG epithelial cell growth, thus validating its potential as a scaffold. Under dynamic culture, LG cells grew more rapidly, eventually completely remodeling the mucosa to form a thick cell layer on the surface of the submucosa, resulting in an increased overall metabolic activity. That larger cell numbers may be achieved sooner using dynamic culture formats is important because manufacture to good manufacturing practice is labor intensive and costly, so shorter production times are advantageous. Decellularized scaffolds such as SIS-Muc may also be remodeled by the host, which is useful because this indicates that the graft may eventually be completely integrated.\textsuperscript{38}

Ultrastructural evaluation of the cells on SIS-Muc using TEM revealed epithelial cell polarization at the superior surface that resembled polarization within epithelial acini in the native LG.\textsuperscript{22} This polarization is important because formation of acini and duct systems is required for effective supply of LG fluid to the ocular surface. Here, we did not observe polarization or acini-like structures throughout the bulk of the engineered tissue, however, counter to the findings of others.\textsuperscript{19,22} This may be due to the comparatively short culture times used in this study, or the lack of deliberate inclusion of other cell types, such as myoepithelial cells and stromal cells, that are present in native LG and may be required for formation of complex acinus structures. This will be evaluated in future studies. In dynamic cultures only, TEM also revealed the presence of numerous multilamellar bodies. Multilamellar bodies are associated with surfactant protein and lysozyme, both of which were found in human and porcine tears and cell culture supernatants (data for surfactant proteins not shown),\textsuperscript{39} but the reason for their differential presence in dynamic versus static cultures is
unclear. TEM also revealed that cells were well connected to each other (tight junctions and desmosomes), required for tissue integrity, and also to the SIS-Muc, which would be required to permit surgical handling during transplantation.

Crucially, epithelial cells on SIS-Muc retained their secretory profile, demonstrated using IHC where acidic and neutral mucins, Rab5D and HexA, remained evident, further validating SIS-Muc as a scaffold for LG tissue reconstruction. Moreover, lysozyme and lactoferrin were also observed in many cells (unlike when they were cultured on plastic), indicating that epithelial cells may be capable of producing lacrimal fluid of normal composition when cultured on SIS-Muc. Generally, cell–matrix interactions are known to affect cell phenotype as cells respond to external stimuli such as stiffness and basement membrane composition. This is also the case for LG epithelial cells, and, more specifically, the choice of matrix has also been shown to impact on LG epithelial cell differentiation. Interestingly, in dynamic cultures where the cell layer produced was thicker, expression of lysozyme and lactoferrin appeared greater within superior cells, further indicating intercellular organization, or perhaps that secretion was directed toward a “lumen.”

We also evaluated the β-hexosaminidase enzyme activity before and after parasympathetic stimulation in our SIS-Muc cultures on days 2 and 9. In healthy LG, the cells respond to stimuli such as temperature change or pain at the ocular surface, and therefore this should also be the case for a reconstructed gland. We saw a decreased response to carbachol over culture time (between days 2 and 9), which has also been described by others. Although the reasons for this remain unclear, insufficient expression of M3 muscarinic receptor having been ruled out, it likely reflects a limitation of the in vitro environment to support cell cultures because, for example, other supporting cell types are absent. Overall, β-hexosaminidase activity was increased in dynamic cultures over static, which probably reflects the increased number of cells present in these cultures. This raises an interesting point as to at which stage of manufacture such reconstructed tissues should be transplanted, but in vivo work would be required to elucidate this. In any case, increased overall function is clearly beneficial in terms of clinical application. Lactoferrin was not detectable within culture supernatants via ELISA, although it was demonstrably present within cells on SIS-Muc using IHC. This may suggest that, although cells were able to produce lactoferrin, they were unable to exocytose/secrete the protein, perhaps due to the lack of ductal architecture within the tissue. The accumulation of multilamellar bodies within cells (particularly in dynamic cultures) supports this hypothesis.

In summary, we demonstrated for the first time that decellularized porcine gut is a suitable scaffold to support LG epithelial cell growth and that these cells are capable of producing LG-specific proteins. Additionally, we confirmed that dynamic cultures promote LG metabolic activity and function. Further in vivo work is required to evaluate the feasibility of using this LG construct for treatment of DES.

Acknowledgments
The authors thank Felicitas Jahnel and Sabine Seggewiss for excellent technical assistance and Elisabeth Wesbauer for assistance with transmission electron microscopy studies. Supported by the Volkswagen Foundation (Lichtenberg professorship of SS).

Disclosure: I. Massie, None; K. Spaniol, None; A. Barbian, None; G. Poschmann, None; K. Stühler, None; G. Geerling, None; M. Metzger, None; S. Mertsch, None; S. Schrader, None

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