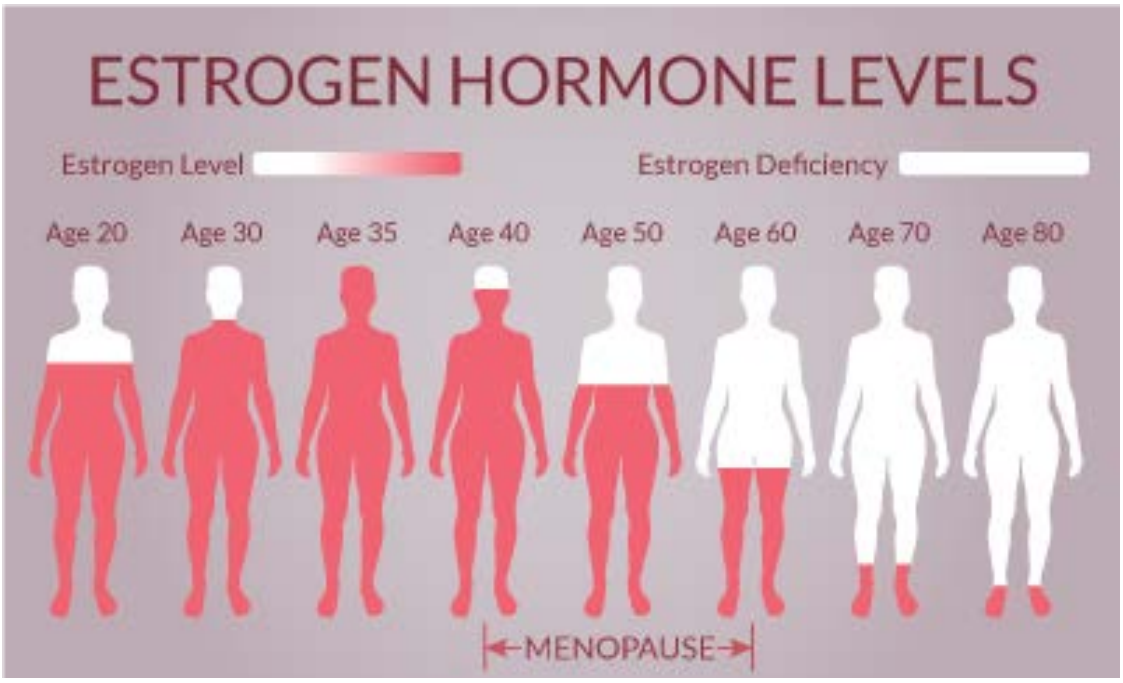
The role of estrogen in primary Sjögren's syndrome salivary gland dysfunction

MSc Internship report



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Abstract

Introduction:

Primary Sjögren's syndrome (pSS) is a chronic autoimmune disease that is characterized partly by salivary gland (SG) dysfunction. Saliva is produced by acinar cells, and transported through the ductal system into the mouth. SG progenitor cells (SGPCs) maintain SG homeostasis, and reside potentially in the basal layer of the striated ducts (BSDs). pSS predominantly manifests in post-menopausal women, where estrogen levels are low. In other systems, estrogen receptor-α (ER-α) stimulation induces epithelial proliferation, and ER-β apoptosis and autophagy. ER-β is expressed at higher levels in the SG than ER-α.

Aim:

Characterize the role of estrogen in the development of SG pathology in pSS, specifically focusing on SGPC and acinar cell biology and ER-β.

Methods:

ER-β expression in BSDs and acinar cells was examined by immunostaining. An AQP5 counterstain identified tissue architecture. Healthy, 'sicca' (SG dysfunction for non-pSS reasons) and pSS parotid SG tissue were analyzed. SGPCs cultured as salivary gland organoids (SGOs) in a self-renewal assay were used to examine effect of estrogen on SGPCs . Expression levels of ER-β and estrogen related genes were examined in a single cell (sc)-RNASeq database.

Results:

Increased ER-β expression in the BSD cells and acinar cells was detected in pSS and sicca tissues, compared to controls. SGPCs treated with estrogen showed enhanced proliferation, (increased SGO formation potential). Gene expression for ER-β was not detected in acinar cells by sc-RNASeq, however expression of autophagy markers p62 and LC3B were lower in acinar cells in the vicinity of infiltrates, indicating a downregulation of autophagy.

Discussion:

Our combined immunostaining and SGO culture results suggest that estrogen signaling may play a role in homeostasis of the SGPCs resident in the BSD layer of

the SG. Clarification of the role of ER-α signaling in this process will be necessary, as ER-β normally induces cell apoptosis. Indeed, increased ER-β expression in the BSDs of the SGs in pSS may indicate increased susceptibility for apoptosis and disruption of SG homeostasis. Acinar cell ER-β expression was not detected by sc-RNASeq, however was observed in immunostaining, perhaps indicating a translation/transcription control axes. Acinar cell ER-β positivity by immunostaining and decrease of autophagy gene expression in acinar cells from SGs with infiltration may also suggest a protective role for estrogen signaling for acinar cells in pSS.

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Introduction

Sjögren's syndrome (SS) is a chronic autoimmune disease that often negatively affects the exocrine glands, which in turn leads to symptoms such as xerostomia, xerophthalmia and vaginal dryness (Stewart et al 2008). This disease has negative impacts on all the aspects of the afflicted patient's life such as reduced physical and social function, lower vitality, poor oral and mental health (Stewart et al 2008). Impairment of the exocrine glands has been found to be accompanied by lymphocytic infiltrations in the affected areas,but is not strongly corelated with loss of saliva production. (McCoy et al. 2019; Pijpe et al 2007; Wang et al 2018).

The salivary gland (SG) consists of acinar cells, which are the site of the production of saliva (Kondo et al 2015). The saliva that is produced is then drained and modified through the ducts, which consists of the intercalated ducts (ID), striated ducts (SD) and lastly the excretory ducts (Figure 1) (Kondo et al 2015).

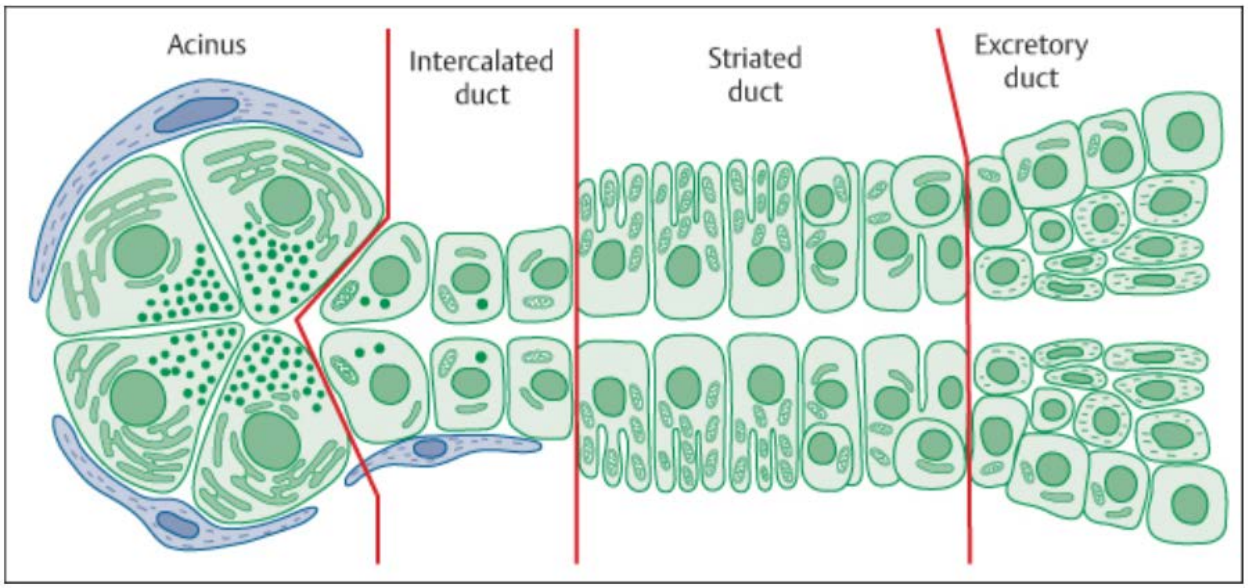

Figure 1. Cellular organization of a functional SG gland unit containing acini and ducts. Adapted from: https://pocketdentistry.com/malignant-salivary-gland-neoplasms-classification/

It has been widely proposed that viral infections may cause the onset of primary SS (pSS) through increase of expression for TLR1, TLR2, TLR3, TLR4, TLR4, TLR7 and TLR9 in SG epithelial cells (SGECs) (Kawakami et al 2007; Youinou et al 2005).In the context of most pSS studies, SGECs refer to cells of the SDs. SGECs in pSS were also observed to be susceptible to detachment-induced-apoptosis (anoikis) through TLR3 ligation (Manoussakis et al 2010). Furthermore, SGECs have been observed to

produce cyto-/chemokines such as IL-6, CXCL13 and CXCL10 in pSS (Kawakami et al 2007; Wang et al 2018). The chemokines in turn have been proposed to attract lymphocytes such as macrophages, dendritic cells, T cells and B cells to the affected area (Ibrahem et al. 2019). Furthermore, SGECs in pSS may further be vulnerable to apoptosis through B cells that are associated with pSS by the activation of protein kinase C delta (Varin et al 2012). The final phases of pSS SG pathology is characterized by an increase of infiltrates comprised of mainly by B cells in the SG (Barone et al 2015). This is in part clinically diagnosed through the use of the focus score (FS) of >1 (alternatively described as a Chisholm FS of 4) which represents an aggregate of 50 or more infiltrates in a $4mm^2$ glandular section (Barone et al 2015). These infiltrates and activated epithelial cells may produce pro-inflammatory cytokines such as interleukin 1 beta(IL-1B), IL-6, IL-12, IL-18, tumor necrosis factor α (TNFα), and B cell activating factor (BAFF) (Roescher et al 2009). These inflammatory cytokines may induce proliferation in resident epithelial cells and consequently cause premature senescence of resident epithelial progenitor cell populations (Pringle et al 2019). Further activation and survival of B cells occurs through the production of BAFF (Ibrahem et al 2019). Germinal centers (GCs) are sometimes found in SGs of pSS patients, formed through the rearrangement and organization of B cells within the affected exocrine gland (Ibrahem et al 2019). Furthermore, formation of lymphoepithelial lesions (LELs), consisting of hyperplastic epithelium infiltrated with T cells and B cells, may also occur (van Ginkel et al 2019). Formation of LELs and GCs , in addition to immune foci, can further replace a large portion of the affected gland and in turn disrupt the glandular structure and causing further dysfunction of the SG.

Interestingly, pSS predominantly manifests in women with a ratio of 9 out of 10 (Qin et al 2015). The onset of pSS generally occurs at the ages when menopause is prevalent (Qin et al 2015). Women with pSS have lower cumulative menstrual cycling and estrogen exposure when compared to controls with sicca (‘dry mouth’) symptoms originating from other, non-pSS causes (McCoy et al 2019). Furthermore, postmenopausal women have lower capacity in producing saliva upon stimulation (Minicucci et al 2013). As menopause is characterized by lower levels of estrogen, it can suggest that estrogen may play a role in preventing the development of SS in women.

Estrogen has been found to interact with two types of estrogen receptors (ER) in epithelial cells (Barone et al 2008; Helguero et al 2005). These receptors are the estrogen receptor alpha (ER-α) and estrogen receptor beta (ER-β) (Barone et al 2008; Helguero et al 2005). Both ERs exhibit opposite functions from the other. Before stimulation through estrogen, both ERs are palmitoylated and are associated with caveolin-1 (CAV) where they are localized at the plasma membrane (Barone et al 2008). Upon stimulation with estrogen, ER-α would be de-palmitoylated and in turn dissociates with CAV, which would lead to a downstream signaling for proliferation. On the contrary, upon stimulation ER-β would increase the association with CAV alongside p38 and promote apoptosis (Barone et al 2008). Interestingly, ER-β is the predominant ER in the acinar and ductal cells of SG (Välimaa et al 2004). This may suggest that ER-β may play a role in maintaining the cellular composition in the SG.

The homeostasis of the SG is thought to be maintained by SG progenitor cells (SGPCs) (Nanduri et al 2011; Nanduri et al 2014; Pringle et al 2016; Pringle et al 2019). Human SGPCs that were transplanted in irradiated SGs *in vivo* in mice were able to restore saliva production (Pringle et al 2016). This indicates the probable capability of human SGPCs to differentiate in acinar cells. The SGSCs are thought to reside in the basal SDs of the SG (Pringle et al 2019). These cells differentiate into IDs and eventually into acinar cells (Pringle et al 2019).

Hypothesis:

Due to the low levels of estrogen from menopause, we hypothesize that either ER-β signaling may play a role in the development of pSS.

Aim:

The effects that estrogen has on the SG has yet to be investigated in pSS, thus the aim of this study is to characterize the role estrogen may play in the development of SG pathology in pSS. To examine whether there is abnormal ER-β expression, the parotid glands of pSS and sicca patients, and healthy donors will be immunostained for ER-β. The protocol for SGPC isolation developed by Pringle et al. was used for organoid culture (Pringle et al 2019). RNASeq was also conducted with pre-existing database to examine whether the expression levels for the ER or estrogen related genes are affected in pSS SG cells.

Material & Methods:

Immunostaining for ER-Beta

Parotid gland tissues from healthy donors, from patients diagnosed with pSS, and from a control group of patients with dry mouth (“sicca”) without signs of autoimmune disease were analyzed.

The paraffin embedded tissues were cut in 4 µM sections and mounted onto microscope slides. Dewaxing and rehydration was conducted by submersion of the slides in the following order: Xylene for 15 minutes, Xylene for 15 minutes, 100% Alcohol for 5 minutes, 100% Alcohol for 5 minutes, 96% Alcohol for 5 minutes, 70% Alcohol for 5 minutes, in demi water for 5 minutes. Antigen retrieval was performed by using a sodium citrate antigen retrieval buffer. Buffer consisted of 10mM sodium citrate tribasic dihydrate and 0.05% Tween-20 (Sigma). The pH for the buffer was corrected to 6.0 and preheated afterwards in a pressure cooker in a microwave for 15 minutes at 700W. The tissue slides were immediately transferred into the buffer and incubated for 12 minutes at 650W. Double staining was performed, using the Lab Vision Polymer Detection (LVPD) system according to instruction of the manufacturer (catalog no. TL-012-MARH, Thermofisher). Tissue slides were double stained for aquaporin-5 (AQP5) to distinguish acinar cells and ER-β.

Primary antibodies were incubated in different times and dilutions. Primary antibody for AQP5 (catalog no. ab215225, clone EPR3747, abcam) was incubated for 1 hour at room temperature in a dilution of 1:400. The primary antibody for ER-β (catalog no. MAB7106-SP, clone 733930, R&D systems) was incubated overnight at 4°C in a dilution of 1:50. Incubation time for the visualization components of LVPD system for ERβ was 10 minutes and 10 seconds for AQP5. Images of the stained slides were taken using a Bx50 microscope and Pixelink Capture software.

Source of SG tissue

Healthy parotid SG tissue samples biopsies were collected from donors who received treatment for oral squamous cell carcinoma. These samples were acquired under the approval of the institutional review board [IRB] and consent from the donors. Elective head and neck dissection procedure was performed that exposed and removed the parotid SG in these patients. The collected tissue is devoid of oral squamous carcinoma cells, as these cells do not migrate to the parotid SG

Isolation of salivary gland organoids:

The healthy parotid SG tissue samples were processed in Hanks' balanced salt solution (HBSS) that contained 1% bovine serum albumin (BSA, Invitrogen). These biopsy samples were digested mechanically by using a gentleMACS dissociator (Miltenyi Biotec) in a buffer solution consisting of HBSS/1% BSA buffer that contained 0.63 mg/mL type II collagenase (Invitrogen), 0.5 mg/mL hyaluronidase (Sigma-Aldrich) and calcium chloride at a concentration of 6.25 mM, for 30 minutes at 37°C. Total volume was adjusted through the following criteria: 40 mg of tissue was processed in 1 mL buffer volume. The digested cells were further isolated through centrifugation, washing twice in HBSS/1% BSA solution and finally passed through 100-µm cell strainers (BD Biosciences).

The cell suspensions were collected by centrifugation and resuspended in salivary gland organoid (SGO) medium. SGO medium consisted of 25 mL Wnt-3a-conditioned medium (50%), 20 mL Dulbecco's modified Eagle's medium/F-12 medium, penicillin/streptomycin antibiotics (Invitrogen) (40%), 5 mL R-spondin conditioned medium (obtained from RSPO1 cell line; AMSBIO) (10%), 20 ng/mL fibroblast growth factor (Sigma-Aldrich), 20 ng/mL epidermal growth factor (Sigma-Aldrich), 12 mg/mL Noggin, 10 mg/mL insulin (Sigma-Aldrich)10 mg/mL N2 (Invitrogen), 5 µM transforming growth factor β inhibitor (catalog no. A8301; ToCris Bioscience), 1 mM dexamethasone (Sigma-Aldrich).

Primary isolate cells numbering a total of 800,000 were resuspended in a mixture of 25 µL of SGO medium and 50 µL basement membrane Matrigel (BD Biosciences). This suspension was deposited in the center of a 12-well tissue culture plate and solidified through incubation at 37°C for 20 minutes. Lastly, 1 mL SGO medium was added per well containing a solidified gel. Primary spheres formed after 3-5 days of culture. These spheres were extracted from the Matrigel through incubation in 1 mg/mL Dispase (Sigma) for 1 hour at 37°C. Primary spheres that were at a minimum size of 50 µm were counted. The sphere count was used to calculate primary sphere yield per mg of biopsy material.

Self-renewal assay with estrogen exposure:

After the extraction of the primary spheres from the Matrigel as described beforehand, the spheres were processed to form single-cells using 0.05% trypsin-EDTA (Invitrogen). The single cells were counted and cell concentrations were adjusted to 4.0 x 10^5 cells per mL SGO medium. Fifty µL of basement membrane Matrigel was then mixed with 25 µL of the aforementioned SGO mixture and deposited in the center of 3 wells of a 12-well tissue culture plate. The culture plate was then incubated at 37°C for 20 minutes to solidify the Matrigel.

After solidifying, 1mL of either SG organoid medium alone (control), or SG organoid medium containing 10nM or 1µM of 17β -estradiol (Sigma) was added. Medium was refreshed with 500µL of relevant medium types every 2-3 days. Organoids had formed 5 days after seeding. After 9-12 days of culture, the formed spheres were extracted from the Matrigel by incubation in 1mg/mL Dispase (Sigma) at 37°C for 30 minutes.

Organoids that were >50µm in diameter were counted and processed to form a single-cell suspension. The organoid formation efficiency (OFE) was calculated with the following formula:

$$\text{OFE (\%)} = \text{Harvested organoids / seeded cells}$$

The cells were dispersed to form single cells using 0.05% trypsin-EDTA (Invitrogen), counted and seeded in Matrigel as described beforehand. The seeding in the Matrigel was then repeated up to a total of 4 passages. Images of the cells were captured at each passage using an Olympus CKX53 microscope and DP2-SAL software.

Single cell RNASeq:

From single-cell suspensions, cDNA libraries were prepared by following the 10x Genomics 3' v3 protocol, and 1 sample per lane. These samples were sequenced on HiSeq4000 with the aim of 150M reads/sample or ≥ per cell.

Chromium Single Cell Reagent kits v.3 were used to perform single-cell capturing and downstream library constructions. Single-cell gel bead-in-emulsion was generated by loading a total of 11 µl of single cell suspension containing between 5000-6000 cells, 40 µl of barcoded gel beads and partitioning oil to Chromium Chip B. Afterwards, polyadenylated transcripts were reverse-transcribed inside each gel bead-in-emulsion. PCR-amplification was performed on full-length cDNA alongside cell barcode identifiers. Sequencing libraries were prepared and normalized afterwards. BGI MGISEQ-2000 or Illumina platform was used to sequence the constructed library, with the aim of 20.000 reads per cell.

Bioinformatics analysis:

The acquired single cell data was processed with Cell Ranger 3.0.2 using the human reference transcriptome from the 10x Genomics website. The data was further processed using the R package Seurat v3.1.4.The cells were filtered based on the following criteria: Per cell, gene count between 100 and 2500, up to a maximum of 15% mitochondrial RNA.

Genes were then normalized according to the tutorial. The 2000 most variable genes were scaled and used further for clustering. The first 10 principal components were used for clustering (resolution 0.2) and UMAP projection. Cluster markers were established using the "FindAllMarkers" function in Seurat (standard settings).

Results:

Increased ER-β expression in striated ducts of pSS parotid salivary gland tissue:

In order to examine the expression of ER-β in the tissue of pSS SGs, immunostaining was performed on healthy, "sicca'' and pSS parotid gland tissue. Sicca control was included as it is characterized by the dysfunction of the SGs in the absence of systemic diseases. Clinical characteristics of patients from which the parotid glands where analyzed from (Figure 2.A-C) can be found in Table 1.

	Subsection D	Subsection E	Subsection F
Chisholm FS	2	4	4
UWS	0.35 mL/min	0.01 mL/min	0.01 mL/min
AA	No AA found	SSA > 240 (positive)	SSA > 240 (positive)

Table 1. Chisholm FS, UWS flow rates and SSA related information of the sicca and pSS parotid gland tissue from Figure 2 and 4. Subsection D derives from a sicca parotid gland tissue. Subsections E-F derive from pSS parotid gland tissue.

ER-β expression was observed in the SDs of the healthy, sicca and pSS parotid gland tissue, but not in the ID (Figure 2). The SDs are composed of two layers of cells, the basal striated duct (BSD) and luminal striated duct (LSD) layers. ER-β expression was observed in both BSD and LSD layers (Figure 2.C-F). The intensity of the ER-β immunostaining was darker in the BSDs and LSDs of sicca (Figure 2.D) and pSS (Figure 2.E, F) when compared to the SD of healthy (Figure 2.A-C) parotid gland tissue, suggesting more ER-b expression in these tissues. From these results and the current knowledge that SGPCs reside, amongst other places, in the BSD of the SDs, it is possible that estrogen signaling may play a role in SGPCs dynamics.

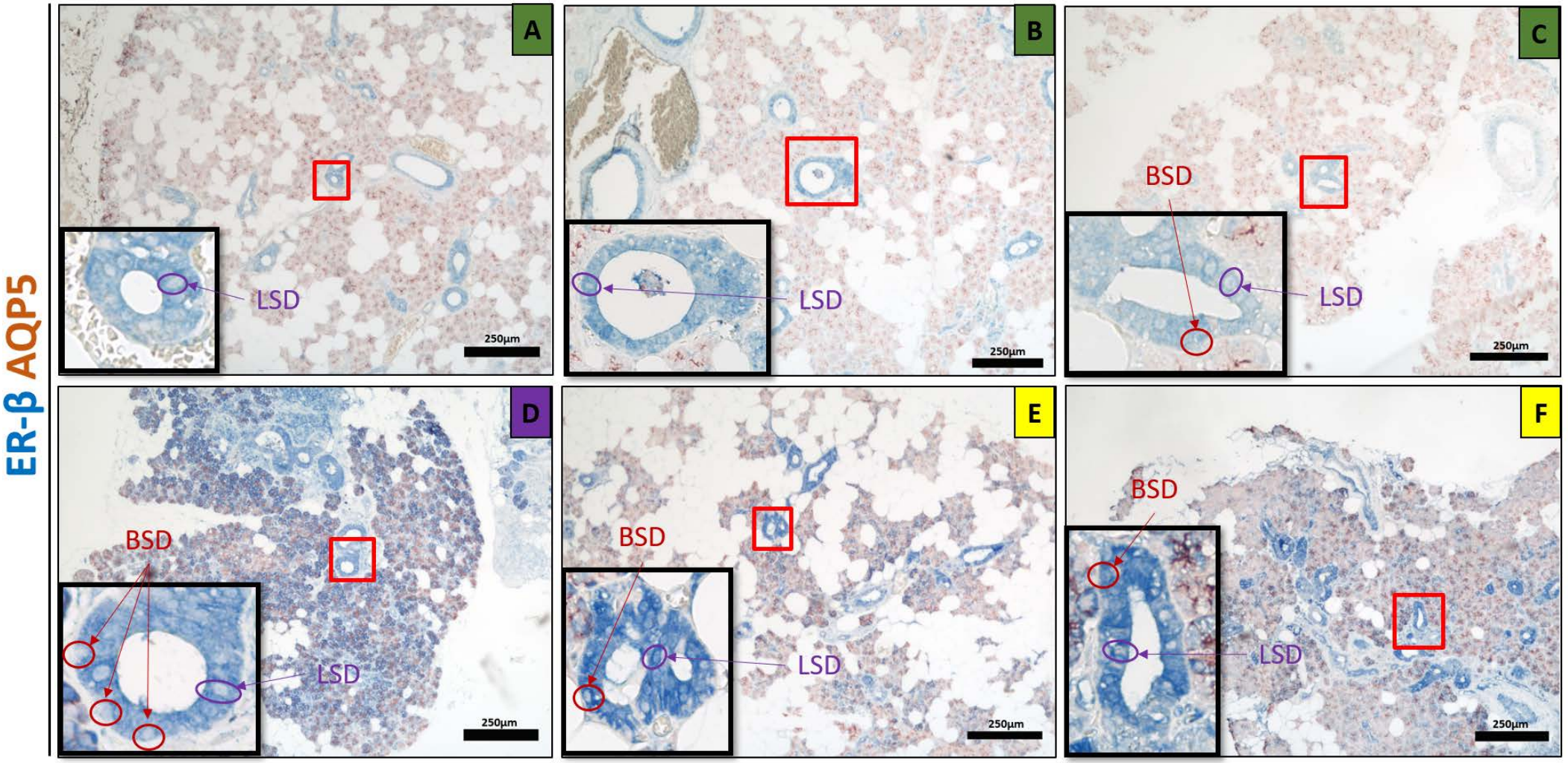

Figure 2. Expression of ER-β is increased in the basal SD (BSD) and luminal SD (LSD) of sicca and pSS parotid gland tissue. AQP5 expression shown in brown and ER-β in blue. High resolution microscopy of striated ducts is shown inset boxes. Examples of BSD cells are circled in red and LSD in purple. A-C derive from healthy parotid gland tissue. D. derives from a sicca parotid gland. E-F derive from pSS parotid gland tissue. A-C. ER-β expression is present in the BSD and LSD of healthy control (HC) parotid gland tissue. Increased ER-β expression in BSD and LSD of pSS (E-F) and sicca(D), indicated by the darker intensity of staining in comparison to HC (A-C).

Increase in regenerative potential shown by salivary gland progenitor cells treated with estrogen:

In order to examine whether estrogen signaling plays a role in the homeostasis of the SGs, SGPCs acquired from healthy donors were grown in organoid cultures and treated with 2 different concentrations of estrogen. In order to examine the long term effect of estrogen on SGPC dynamics, treatment was maintained up to the end of passage 4 (9-12 days per passage) in a self-renewal assay. The SGOs were divided in matched control cultures and SGOs that were treated with either 10nM or 1 μM estrogen. Doses were based on the knowledge that 10nM estrogen increases proliferation for epithelial cells in other studies (Öner et al 2016). Medium was refreshed every 2-3 days up to the end of each passage.

A trend for increase in size and number compared to matched control was observed in the SGOs that were treated with 10nM estrogen at p2 (Figure 3.A). Quantification of raw data showed no difference in OFE between control and SGOs treated with either 10nM or 1μM (Figure 3.B). When the data was normalized to matched control for each patient however, SGOs treated with both doses of estrogen increased in OFE at p2 (Figure 3.C). Since the *n* numbers were low, no statistical analysis could be conducted. As BSD cells are likely to be the predominant progenitor cell type in in SGO cultures, and in addition to our observed ER-β expression in the BSD layer (Figure 2.C) , estrogen may play a role in BSD progenitor cell regenerative capabilities.

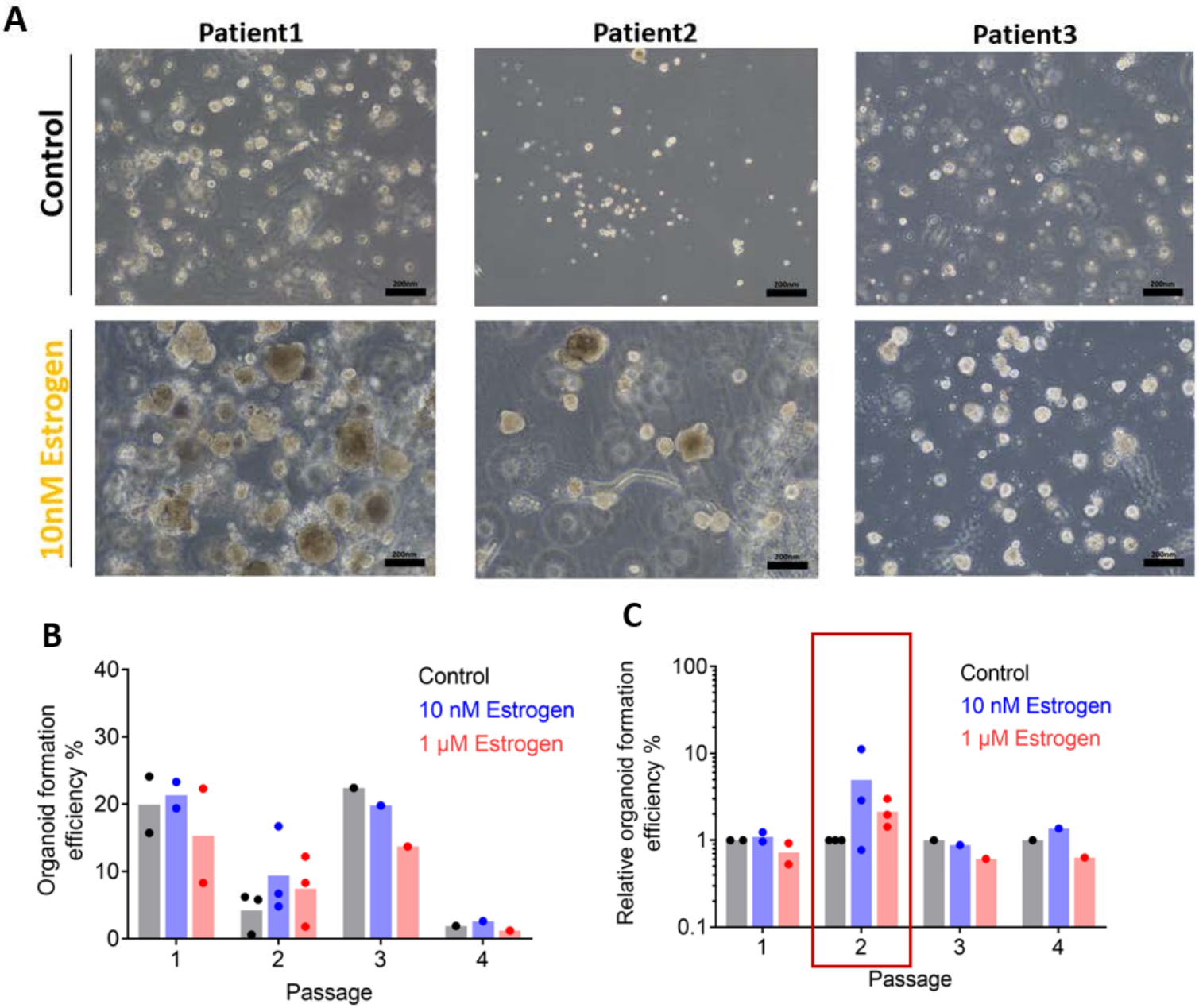

Figure 3. Increased regenerative potential of SGOs treated with estrogen. A. Phase-contrast of organoid culture at passage 2 of the 3 healthy control (HC) treated with and without 10nM estrogen. B. OFE of HC treated with 10nM, 1uM or no estrogen. Individual points represent a separate patient culture. C. Normalized OFE (scaled to Log10) of HC treated with 10nM, 1uM or no estrogen. Individual points represent a separate patient culture. Increase in normalized OFE is highlighted at p2.

Increased ER-β expression in acinar cells of pSS parotid gland tissue:

In order to examine the expression of ER-β in the acinar cells of pSS SGs, immunostaining was performed for healthy, sicca'' and pSS parotid gland tissue. Clinical characteristics of patients from which the parotid glands where analyzed from can be found in Table 1 (the same patients as Figure 2).

Minimal ER-β expression was observed in the acinar cells of the healthy parotid gland tissue (Figure .4A-C.). Expression was notably higher in sicca (Figure 4.D) and pSS (Figure 4.E, F) acinar cells. From these results, increase in the expression of ER-β in acinar cells may be a marker for SG dysfunction, not limited to pSS, but applicable to other SG dysfunction disorders.

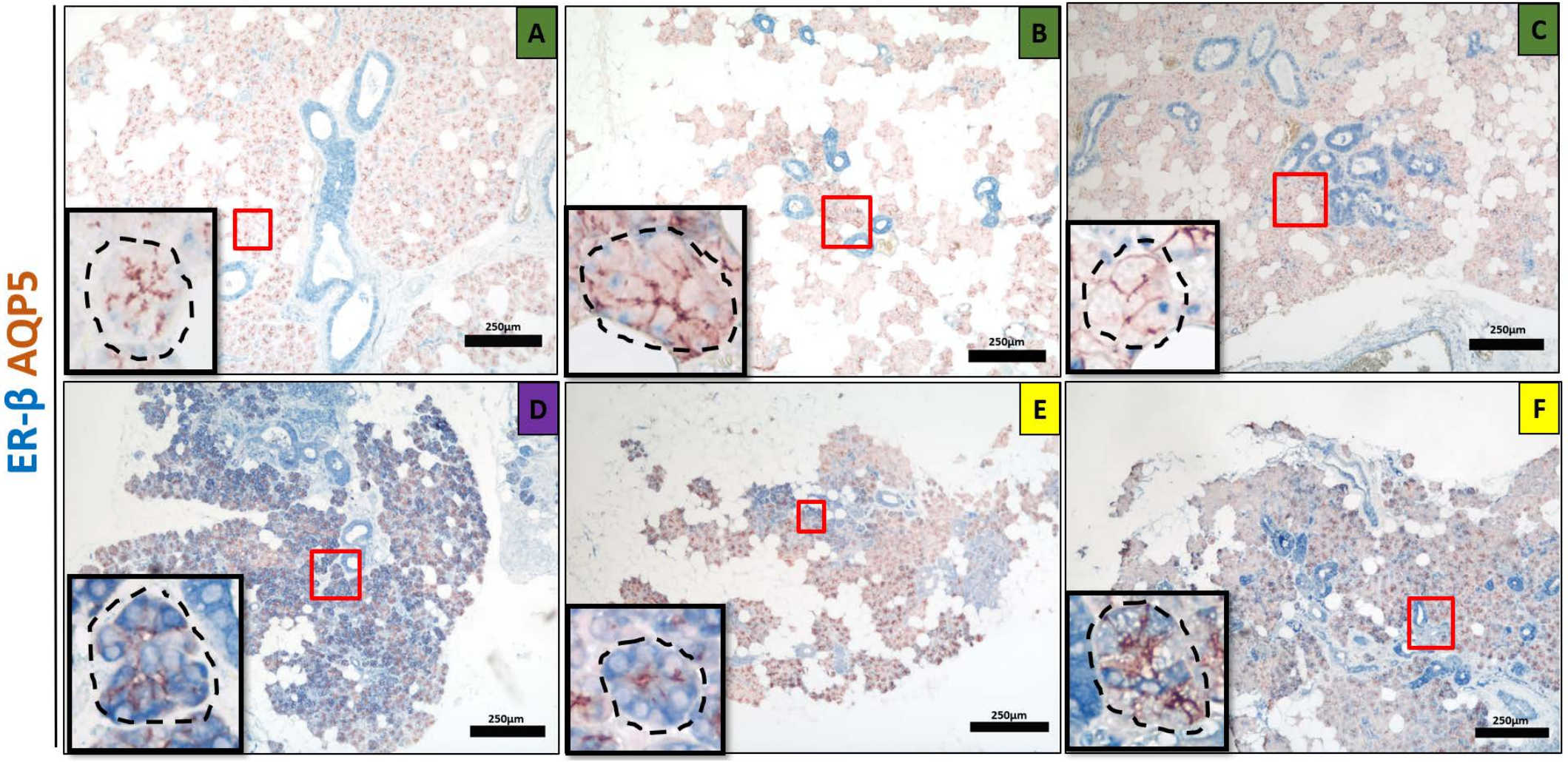

Figure 4. Expression of ER-β is increased in the acinar cells of sicca and pSS parotid gland tissue. High resolution microscopy of acinar cells is shown inset boxes. A-C. derive from healthy parotid gland tissue. D. derives from sicca parotid gland tissue. E-F derive from pSS parotid gland tissue. AQP5 expression is shown in brown and ER-β in blue. Minimal ER-β expression is present in the acinar cells of HC parotid gland tissue in A-C. Increased ER-β expression in acinar cells of sicca (D) and pSS (E-F), indicated by the darker intensity of staining in comparison to HC (A-C).

Lower expression levels of autophagy markers in a subgroup of acinar cells

In order to further investigate the potential role estrogen signaling may have in acinar cells, a pre-existing single cell (sc)-RNASeq database was examined.

Single cells that were isolated from biopsies of SGs from a HC and 3 pSS patients were used for this database. The pSS patients differed in the degree of lymphocytic infiltration in their SGs, reflected in an increasing focus score (FS). Cells isolated from biopsies were grouped on basis of transcriptomic similarities. Existing knowledge of genes characteristic of different cell types was used to assign a cell identity to the different cell clusters obtained. A group of acinar cells were identified, based on gene expressions related to acinar cells of the SG (Figure 5A). Furthermore, this group of acinar cells was composed of two subgroups based on the differences in their transcriptional profiles (cluster 0 and cluster 1, Figure 5B). Cluster 0 were acinar cells, predominantly derived from SGs with a FS of 0 (Figure 5B, C). Cluster 1 was composed of acinar cells, predominantly derived from SGs with a FS of 0-1 (Figure 5B, C).

Gene expression for ER-β was not detected in acinar cells with the FS of 0 and 0-1 (Figure 5D). Apart from inducing apoptosis however, ER-β has also been found to inhibit the expression of autophagy inhibitors (Zieljnok et al 2014). This inhibition may thus promote autophagy. Interestingly, there is evidence in literature that autophagy is downregulated in the acinar cells of pSS patients (Barrera et al 2019). Based on this knowledge, gene expression for autophagy markers LC3B and p62 were examined (Figure 5E, F).

Gene expression for the autophagy markers LC3B and p62 in acinar cells was detected to be lower in the presence of infiltrates (Figure 5E, F). This may suggest that autophagy is downregulated in acinar cells that are in the vicinity of infiltrates.

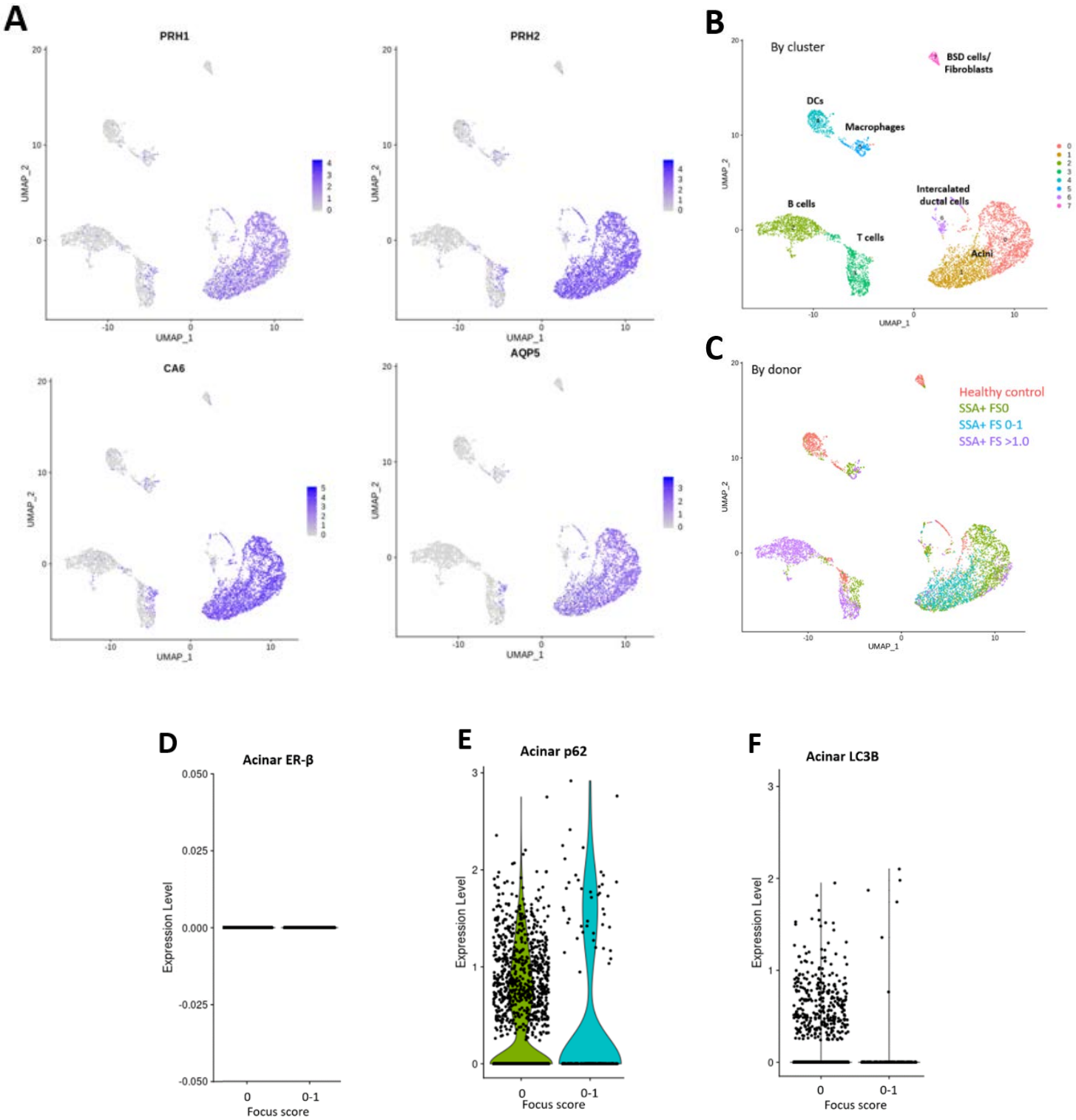

Figure 5. No gene expression for ER-β detected in the two subgroups of acinar cells. Gene expression for the autophagy markers were decreased in the vicinity of infiltrates. A. t-SNE plots of genes that are likely to be expressed by acinar cells in the SG. B. t-SNE plot of cell type grouping. Acinar cells can be separated in two subgroups, acinar cells derived from SG tissue with a FS of 0 and 0-1. C. t-SNE plot sorted by healthy control donor and donor with a FS of 0, 0-1 and >1. D. Gene expression levels for ER-β in acinar cells from SG tissue with FS 0 and 0-1. E. Gene expression levels for autophagy marker p62 in acinar cells from SG tissue with FS 0 and 0-1. F. Gene expression level for autophagy marker LC3B in acinar cells from SG with FS 0 and 0-1.

Discussion:

The autoimmune disease primary pSS occurs predominantly in women, commonly around the age of postmenopausal state (Qin et al 2015). Postmenopause can be characterized by the decline of estrogen levels in women. (Qin et al 2015). Despite this however, the role of estrogen in pSS development has yet to be thoroughly investigated. In this study the role that estrogen may play was examined in the acinar cells and ductal cells of SGs in pSS.

Role of estrogen signaling in ductal progenitor cells

SGPCs are thought to maintain the cellular homeostasis of the SG through their proliferation and differentiation into fresh, saliva producing acinar cells (Nanduri et al 2011; Nanduri et al 2014; Pringle et al 2016; Pringle et al 2019). SGPCs are viewed to be located in the BSD layer, and IDs amongst other niches (Lombaert et al 2008; Pringle et al 2013). SGPCs can be studied in vitro through culture of SGOs (Lombaert et a. 2008; Pringle et al 2016; Pringle et al 2019). Exposure of human parotid SGOs to estrogen in our self-renewal assay, which assesses SGO cell proliferation, induced an increase in organoid formation efficiency. SGOs were confirmed in a recent study to express keratin-14 (K14) (Pringle et al 2019), and contain a minority of cells also expressing keratin-7 (K7) (manuscript under review). According to immunohistochemical staining, K14+K7- cells are likely to represent SDs in the basal layer, whereas K14+K7+ represent IDs (Alam et al 2011; Kwak et al 2016). Furthermore, it was observed in this study that ER-β expression was present in the BSDs of healthy parotid gland tissue (Figure 2C). These three observations (increased organoid formation efficiency with estrogen, domination of SGOs by likely BSD cells and BSD cell expression of ER-β) would suggest that BSD cells may respond to estrogen exposure by proliferation, and that estrogen signaling plays a role in BSD SGPC biology.

In another study, ER-β has been reported to be the predominant estrogen receptor in the SG, in comparison to ER-α expression (Välimaa et al 2004). Estrogen signalling is known to cause an increase in proliferation in epithelial cells through estrogen ER-α engagement, or apoptosis through ER-β signaling (Dall et al 2018; Helguero et al 2005; Treeck et al 2010). When taking our results into account, whereby estrogen

application resulted in increased SGO formation efficiency, this may suggest that ER-α signaling still occurs in BSD cells, despite ER-β expression being predominant in the SGs. Estrogen has an equal binding affinity for both ER-α and ER-β receptors (Zhu et al 2006). As increase in proliferation was observed in the SGOs in our study, this may suggest that ER-α expression is higher than ER-β in the PCs that mainly consists of BSDs. The expression of ER-β in the SG in general is dominated to that of ER-α, however it has yet to be examined whether this is the case in BSDs specifically (Välimaa et al 2004). Thus, the examination of ER-α expression in the BSDs of the SG is still necessary to further shed light on whether estrogen may increase proliferation of SGPCs.

SGPCs have been shown to be reliant on several signaling pathways for their function, such as Wnt and EGF signaling pathways (Maimets et al 2015; Nanduri et al 2014). Epidermal growth factor (EGF), for example, was included in the SGO culture medium. When mammary epithelial cells (MEC) were treated with agonists specific for ER-α and ER-β, an increase in proliferation or apoptosis respectively was observed, in line with commonly accepted downstream fucntiosn of the two receptors (Cotrim et al 2013). When MECs treated with ER-β were co-incubated with EGF however, induction of apoptosis was repressed (Cotrim et al 2013). EGF is known to activate extracellular kinase 1 and 2 (ERK1/2) (Cotrim et al 2013; Lujan et al 2010). When ERK1 was inhibited in the MECs, induction of apoptosis through ER-β was completely restored (Cotrim et al 2013). This may suggest that EGF in the SGO culture in our study may have repressed the induction of apoptosis caused by ER-β signaling through ERK1 signaling. Furthermore, in our study Wnt3a-conditioned-medium was also included in the SGO culture medium. Mesenchymal progenitor cells that were treated with Wnt3a showed an upregulation in ER-α expression and downregulation in ER-β expression (Gao et al 2013). This mechanism in which the change of ERs expression occurs has yet to be explained. In our study, expression for ER-α and ER-β in the SGO cultures was not examined. The downregulation of ER-β however, may have still occurred in the SGO which is indicated by the increase in proliferation when treated with estrogen despite ER-β being predominantly according to literature.

Lastly, we observed that ER-β expression was increased in the BSDs of pSS in comparison to BSDs in healthy parotid gland tissue (Figure 2E, F). In other studies, pSS ductal cells in general were more vulnerable to apoptosis, with no specific

mention of the BSD layer. (Nakamura et al 2018; Varin et al 2012). Taken together with the increased ER-β expression in BSDs, these data may suggest that BSDs in pSS are also vulnerable to apoptosis. As described before, SGPCs are also located in the BSDs, which are thought to be responsible in keeping the cellular homeostasis in the SG (Nanduri et al 2011; Nanduri et al 2014; Pringle et al 2016; Pringle et al 2019). Thus, the increased ER-β expression in BSDs may play a role in the development of pSS pathology through disrupting this cellular homeostasis.

The role of estrogen signaling in salivary gland acinar cell biology

The expression of ER-β was observed to be increased in acinar cells of pSS in comparison to healthy control. Similar to the BSD layer, since ER-β signaling leads to apoptosis, this may suggest that these acinar cells in pSS are more vulnerable to apoptosis, as seen in other studies regarding pSS (Nakamura et al 2018; Varin et al 2012). Due to the postmenopausal decline in estrogen levels however, acinar cell apoptosis by ER-β signaling in pSS may be less likely to occur. This may be consistent with the apoptotic acinar cells in pSS that are unable to undergo apoptosis due to anti-apoptotic factors such as Bcl2 (Kong et al 1998). These apoptotic acinar cells may in turn prevent the formation of new functional acinar cells that may form from the BSDs.

In addition to its role in the induction of apoptosis and involvement in cellular signalling pathways such as EGF and Wnt, ER-β signaling is also involved in the process of autophagy. ER-β signaling is capable of promoting autophagy through the inhibition of autophagy inhibitors (Zielniok et al 2014). ER-β expression in acinar cells was not detected in sc-RNASeq, however was observed in the immunostaining, suggesting that there is a control between translation and transcription. The gene expression levels for the autophagy markers LC3B and p62 were lower in acinar cells in the vicinity of infiltrates in our study (Figure 5F, G), suggesting that autophagy is downregulated. Pro-inflammatory cytokines IL-6, IL-1β and IL-8 amongst others were observed to be upregulated alongside a decrease in autophagy markers such ATG-5 and Beclin1 in acinar cells in SGs of pSS patients (Barrera et al 2019; Roescher et al 2010; Sakai et al 2008). 3D-acini that were deficient in autophagy were observed to also have increased mRNA levels of IL-6, IL-1β and IL-8, suggesting that autophagy as an anti-inflammatory mechanism (Barrera et al 2019). Interestingly, pro-inflammatory

cytokines may also affect the estrogen levels (Capellino et al 2014). Aromatose activity may be stimulated by pro-inflammatory cytokines, which in turn induce the conversion of androgens in the affected area into estrogens (Capellino et al 2014). This may upregulate ER-β signaling, and potentially maintain autophagy flux. Furthermore, androgen levels have been observed to also be lower in postmenopausal women (Yasui et al 2012). The occurrence of menopause may limit ER-β signaling through lower levels of estrogen and androgen.

As pSS occurs predominantly in women around the age of postmenopausal state, when estrogen production is decreased, estrogen may play a role in maintaining autophagy by inhibiting the inhibitors for autophagy. Before menopause occurs, ER-β signaling in the acinar cells could lead to the inhibition of the autophagy inhibitors (Figure 6A). Furthermore, pro-inflammatory cytokines may stimulate ER-β signaling by inducing the conversion of androgens into estrogens, thus increasing the estrogen levels. Through this process, the activity of autophagy may be maintained partly by ER-β. In the case of postmenopause however, decline in estrogen and androgen levels leads to the lack of ER-β signaling. This can lead to the downregulation of autophagy as ER-β signaling is lower (Figure 6B). Since there is an increase of inflammation in pSS patients, the increased expression of ER-β in acinar cells may occur in an attempt to decrease inflammation through upregulation of autophagy. Due to the lack of ER-β signaling however, autophagy would remain downregulated (Figure 6B). Thus, we hypothesize that estrogen may play a role in modulating autophagy in pSS by inhibiting the inhibitors for autophagy.

Increase in ER-β expression in the BSDs of pSS were also observed. As BSDs play a role in maintaining the cellular homeostasis of the SG, dysfunctional acinar cells may also be caused by impaired BSDs. Whether ER-β signaling and autophagy may play a role in BSDs of SGs in pSS however still needs to be investigated (BSDs could not be confidently identified in our sc-RNASeq dataset).

In conclusion, ER-β signaling may play a role in SG homeostasis through preventing the inhibition of autophagy in acinar cells, also in response to proinflammatory cytokines. Furthermore, estrogen may increase proliferation of SGPCs that are present in the BSDs and play a role in cellular homeostasis of the SG. Increased ER-

β expression in the BSDs of the SG in pSS may indicate increased susceptibility for apoptosis and thus disruption of the cellular homeostasis in pSS.

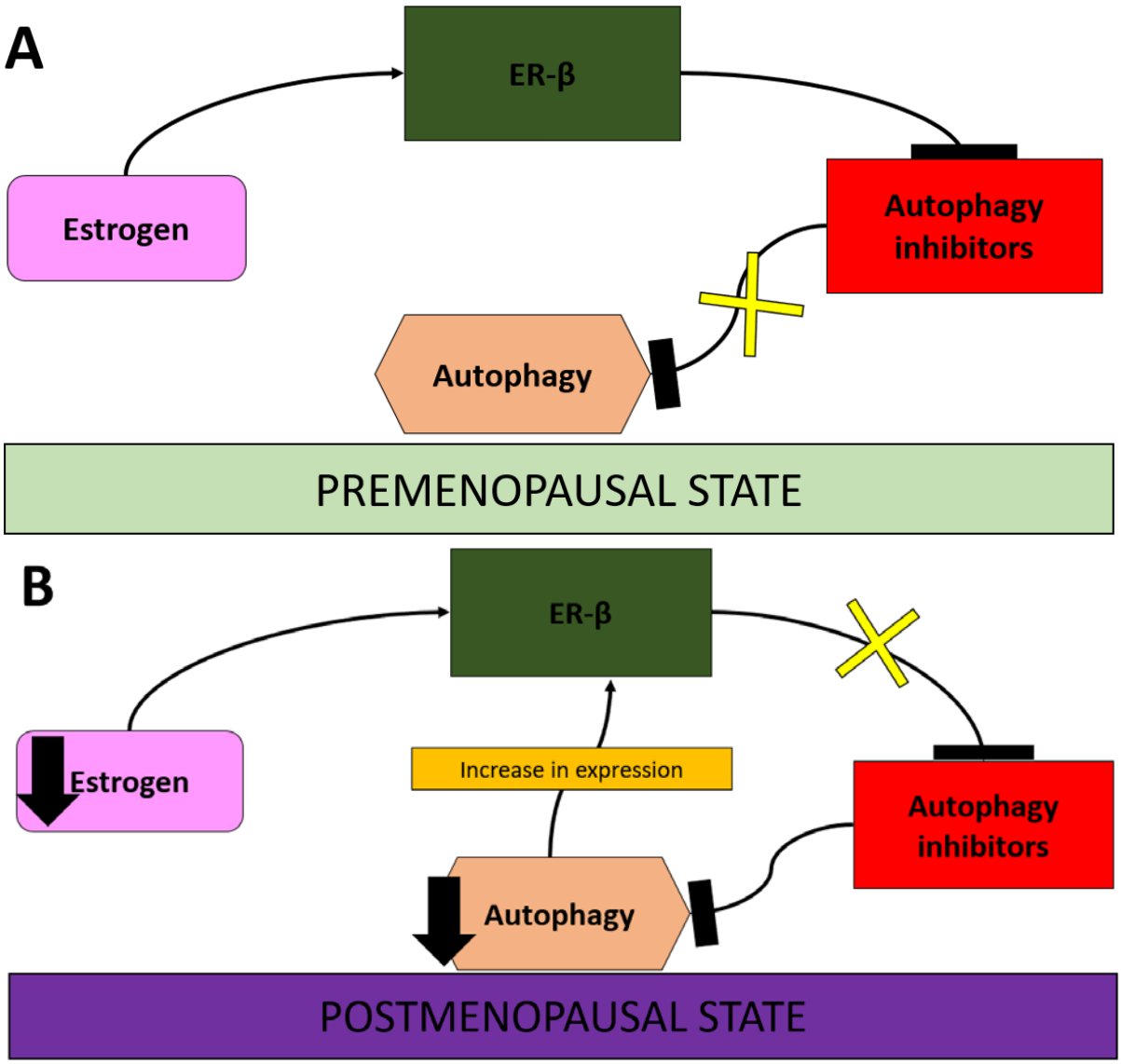

Figure 6. Hypothetical schematic overview of ER-β signaling preventing the downregulation of autophagy in premenopausal state and inability to do so in a postmenopausal state due to lack of estrogen. A. In a premenopausal state, ER-β signaling occurs which may lead to the inhibition of autophagy inhibitors and prevent the downregulation of autophagy. B. Due to the lack of estrogen in the postmenopausal state, ER-β signaling cannot occur which may lead to the inhibition of autophagy through autophagy inhibitors. In response, ER-β expression may increase in an attempt to inhibit the autophagy inhibitors.

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