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Identification of potential effectors in Sjögren’s syndrome using epithelial progenitor cells of the salivary gland

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HENRY M. GOLDMAN SCHOOL OF DENTAL MEDICINE

THESIS

IDENTIFICATION OF POTENTIAL EFFECTORS IN SJÖGREN'S SYNDROME USING EPITHELIAL PROGENITOR CELLS OF THE SALIVARY GLAND

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DEDICATION:

I would like to dedicate my work to my loving family. My father, Nitin Patel, mother, Neeta Patel and brother, Karan Patel. Without whose constant support and encouragement this would have not been possible.

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Lastly, I would like to extend my thanks to my fellow laboratory colleagues at Oral biology, Boston University who assisted me throughout this year of research.

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ABSTRACT

Sjögren's syndrome (SS) is an autoimmune disease that affects predominantly the salivary glands and lacrimal glands, leading to dry mouth and dry eyes. Although significant effort targeting the activated immune cells in SS has been made, there is still no cure for SS. These observations implicate that immune cell may not be the unique source of the cells that drive the pathogenesis of SS. Recently, Senoo lab has made a novel observation that mice conditionally expressing the transcription factor p63 in progenitor cells of the salivary gland epithelia develop SS-like autoimmune disease. In this study, we investigated whether epithelial progenitor cells of the salivary glands in patients with SS express higher levels of p63 than those in non-disease controls. Our data show that expression of p63 in ductal epithelia of labial salivary glands in patients with SS was significantly higher than that of non-disease controls. Subsequently, we aimed at identifying the potential effectors that drive the pathogenesis of SS by comparing the gene expression profile of the p63-positive salivary gland epithelial progenitor cells of patients with SS and of non-disease controls. To address this issue, we employed novel culture of epithelial stem/progenitor cells we have established recently and used deep-RNA sequencing followed by

verification of differential gene expression using quantitative PCR (q-PCR). Further, we cross-referenced our dataset with known SS-associated genes in literature and narrowed down to the top 11 candidate genes. Future studies should focus on the functional analysis of each p63-assocciated candidate gene in the pathogenesis of SS.

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INTRODUCTION

Sjögren's syndrome (SS), first described in 1933 by the Swedish physician Henrik Sjögren, is classified as an autoimmune disorder of exocrine glands, leading predominantly to dry mouth (xerostomia) and dry eyes (keratoconjunctivitis sicca). The disease primarily affects women, with a ratio of 9:1 over the occurrence in men. There is large variability in disease expression, an insidious onset and resemblance with many other conditions thereby making it misdiagnosed or undiagnosed. Primary SS is not accompanied by any other autoimmune disease, while secondary is accompanied by other conditions like rheumatoid arthritis, systemic lupus erythematosus (SLE), dermato-myositis, psoriasis and others (5).

Oral manifestation of symptoms

SS holds significant importance in the oral cavity. The disease has several oral symptoms of clinical importance from a dental perspective. Oral manifestation includes – oral dryness, impaired speech, eating difficulty, pain and swelling of salivary and ocular glands. These symptoms occur alone or in combination. Oral pain may be either result of hypersensitivity of oral mucosa dental caries, susceptibility to opportunistic infection or acute sialadenitis. Chronic or recurrent conditions have predisposing factors. Further, prescribed xerogenic drugs that suppress salivary gland function like antihistaminic, antihypertensive and psychotropic drugs add to the already existing condition (11). Although the clinical manifestation can determine the condition, underlying mechanisms of the disease process need further investigation. Once identified, the pathway that plays a key role in the pathogenesis of SS can be altered by pharmaceutical drugs.

Histology of the salivary glands

Salivary glands have three types of duct system. Intercalated ducts contain low cuboidal epithelium and narrow lumen. Straited ducts contain low cuboidal epithelium and narrow lumen. Excretory ducts have cuboidal epithelium with stratified squamous epithelium lining the terminus. The acini end pieces may be either of serous or the mucous type: Serous secreting cells are arranged in rough spherical form. Mucous secreting cells are arranged in tubular form. Acinar cells represent about 80 % of the gland mass. Duct cells secrete numerous proteins with important biological activities e.g., nerve growth factor, epidermal growth factor and immunoglobin A (2).

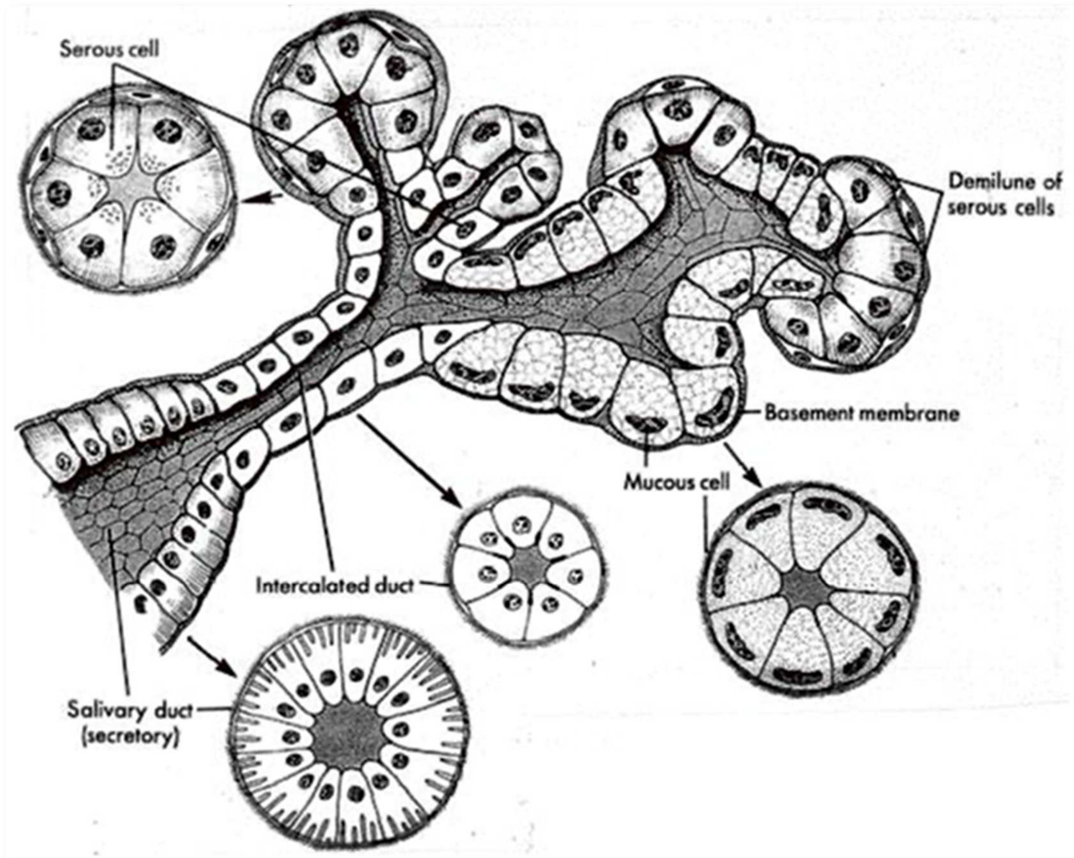

Figure 1: Histological variations in salivary gland ductal cells. The Figure illustrates anatomical location of the main secretory ducts and intercalated ducts in minor salivary glands. There are three types of cells present in these ducts, serous, mucous and demilune of serous cells.

Embryonic development of the salivary glands

Salivary glands originate from an epithelial placode during embryonic development between the 4th and the 12th embryonic weeks in humans.

The embryonic development starts with an epithelial placode, which, in turn, invades the mesenchymal layer and branches up. Simultaneously innervation and vascularization advance with the growth of salivary glands primordia. This directly regulates its maturation. Myoepithelial cells arise from the external epithelial layer and develop into the contracting unit involved in the regulation of secretion. They will then acquire smooth muscle characteristics and locate in direct contact with the acinar structure to regulate the release of secretion (10).

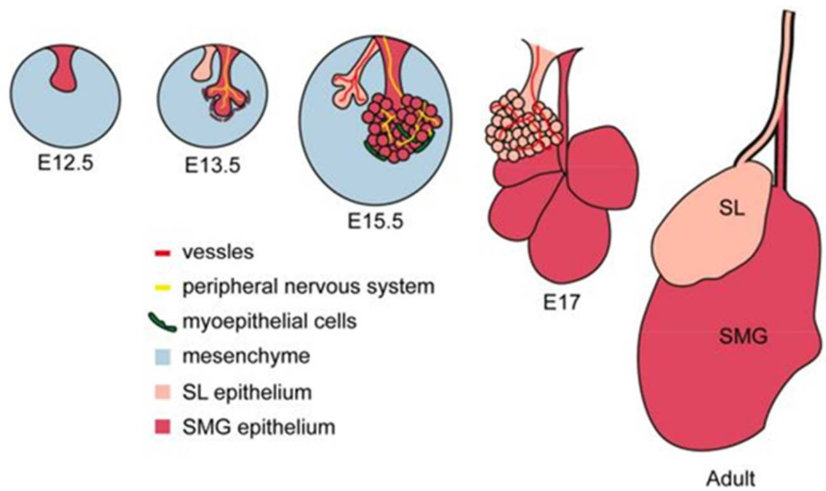

Figure 2 – Embryonic development of murine submandibular glands (SMG) and sublingual (SL) glands. (Porcheri & Mitsiadis, 2019).

SS-associated hyperactivation of lymphocytes in the salivary glands

B-cell hyperactivity is an important feature of the pathogenesis of SS and alterations of B-cell subtypes are also detected in patients with primary SS. Although the pathogenesis of the disease remains largely unknown, the role of the B-lymphocytes appears to be essential in the initiation of the disease. Members of the TNF superfamily (such as BAFF/APRIL) are produced not only by patrolling immune cells but also by the epithelial cells of the salivary glands. Through these pathways, B-cells are activated and start to proliferate in an uncontrolled manner (3). Their pivotal role includes infiltration of the salivary glands to produce an ectopic germinal center and local secretion of autoantibodies. The germinal center can grow independently from the surrounding tissue and can evolve in more complex diseases such as non-Hodgkin lymphoma. (4)

CD80/CD86 and their receptors B7 receptor family play an important role in regulating T cell activation. (26) Expression of CD86 and CD80 were found in ductal and acinar regions in the salivary glands of SS patients but not in those of patients with nonspecific sialadenitis, highlighting the significance of the CD86/CD80-medated T-cell activation in the pathogenesis of SS (7). The functional expression of CD86 on SS patient's salivary gland epithelial cells interact with CD28 and its binding to CTLA-4 was reduced (6). Thus, these cells are functioning to promote production of IL-2 and T-cell proliferation, leading to the increase of the immune response.

Role of the transcription factor p63 in homeostasis and pathogenesis of epithelia

The transcription factor p63 belongs to a family of the tumor suppressor p53. Unlike p53, however, mutations in p63 in human cancers are very rare. Instead, p63 regulates several important cellular and biological processes, including self-renewal, proliferation, and differentiation of stem/progenitor cells of epithelia. Mutations in p63 are associated with ectodermal dysplasia, and cleft lip/palate syndrome 3 (EEC3); split-hand/foot malformation 4 (SHFM4); ankyloblepharon-ectodermal defects-cleft lip/palate; ADULT syndrome (acro-dermato-ungual-lacrimal-tooth); limb-mammary syndrome; Rap-Hodgkin syndrome (RHS); and orofacial cleft 8. The role of p63 in controlling the integrity of stratified epithelial tissues, especially how p63 mutations are associated with the developmental disorders, remains largely unexplored (12).

Preliminary findings in Senoo lab that led to the hypothesis of this study

Senoo lab found recently that transgenic (Tg) mice conditionally expressing p63 in salivary gland epithelial cells upon crossing with cytokeratin 14 (K14)-positive basal epithelia-specific Cre-Tg mice (hereafter p63Tg mice). Specifically, p63Tg mice develop all major SS-associated characteristics, including inflammatory infiltrates, autoantibody production, and hyposalivation. These data indicate that p63Tg mice represent a novel mouse model of SS-like autoimmune disease. Based on these preliminary data, we hypothesize that aberrant activity of ductal epithelial progenitor cells mediated by increased p63 expression contributes to the pathogenesis of SS.(13)

SPECIFIC AIMS:

We will investigate whether epithelial progenitor cells of the salivary glands in patients with SS express higher levels of p63 than those in non-disease controls. In addition, we aim at identifying the potential effectors that drive the pathogenesis of SS by comparing the gene expression profiles of the p63-positive salivary gland epithelial progenitor cells of patients with SS and of non-disease controls.

MATERIALS AND METHODS:

Tissue culture: Establishing primary culture of salivary gland cells.

Human minor salivary glands were obtained from Tufts Medical Center, in a collaboration with Dr. Athena Papas. Six glands were biopsied from each of six subjects (3 Sjogren and 3 Control). Two of six glands were fixed, and the other four glands were used for tissue culture. Each gland was treated individually. Before tissue digestion, the glands were dipped for 10-20 seconds in Isogin (Meiji-Seika, Tokyo Japan) and washed three times in F12 media (Corning, #10-013-CV). Glands were minced with scissors and digested for 1hr at 37°C with frequent agitation in a F12 solution containing 1mg/ml Collagenase (Worthington, #LS004196), 75 U/ml DNase (Worthington, #1189700), 1% Penicillin/Streptomycin (Gibco, # 15070-063) and 0.5ug/ml Fungizone (Gibco Invitrogen). Thereafter, the digested tissues were washed three times in PBS. Before the last spin, each gland was divided in 3 aliquots and plated in one of the three media: FAD, modified Williams-E (ThermoFisher Scientific, #32551-020)(see Figure 3).

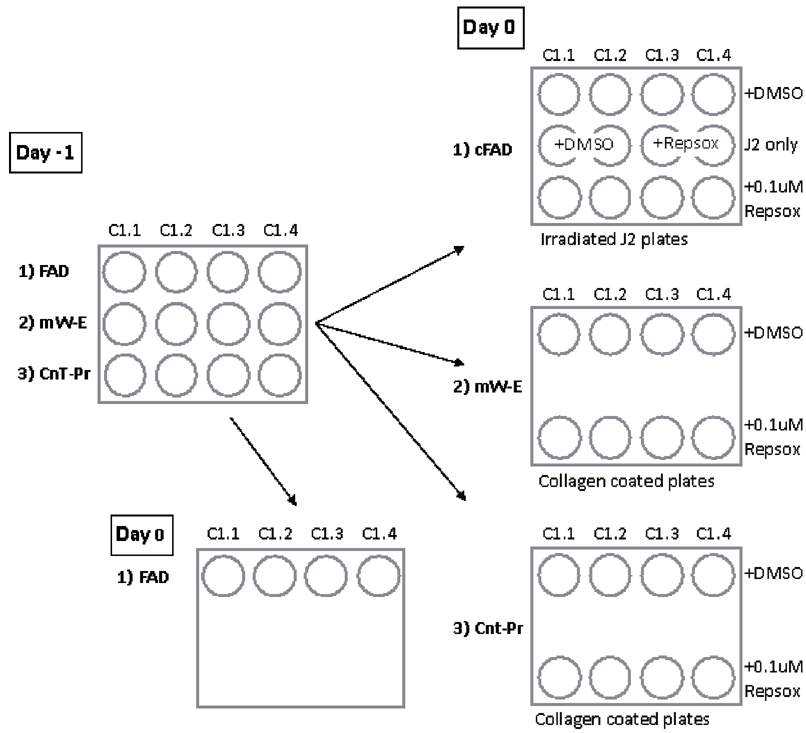

Figure 3 - Plating strategy for the establishment of the salivary gland epithelial progenitor cells.

The cells from CnT-Pr cultures were used for DNA microarray analysis. To perform clonogenic culture, epithelial cells were grown on 3T3- J2 mouse fibroblast cells in FAD medium (DMEM/ Ham's F12 3:1) supplemented with 10% fetal bovine serum, 10U/ml penicillin, 100 ug/ml streptomycin, 5ug/ml insulin, 10ng/ml EGF, 2 x 10-9 M 3'3',5-triiodo-L-tyronine, 0.4ug/ml hydrocortisone, 24ug/ml adenine, and 1x 10-10 M cholera toxin.(9).

Epithelial cells harvested from salivary gland tissue and RNA sequencing.

The epithelial cells grown in CnT-Pr and fibroblasts grown in FAD media were harvested with Trizol (Gibco Invitrogen) and RNA was extracted according to the manufacturer's instructions.

RNA sequencing-

The procedure carried out is from patient purified epithelial cells and fibroblast cells. They were harvested from salivary gland tissue and grown in prime media and FAD respectively. The samples included in the study are listed. The experiment is comprised of 12 samples, profiling p63+ epithelial progenitor cells or fibroblasts obtained from the labial salivary gland of healthy control subjects and patients with SS, The samples were annotated as follows with regard to the subject and biopsy from which each was obtained:

Sample	Subject	Biopsy	Cell type
S3.1 E	SS3	S3.1	EPC
S3.5E	SS3	S3.5	EPC
S3.5Fb	SS3	S3.5	Fb
S4.5Fb	SS4	S4.5	Fb
S5.5Fb	SS5	S5.5	Fb
S5.6E	SS5	S5.6	EPC

Table 1: SS samples established from the minor salivary glands of patients.

Sample	Subject	Biopsy	Cell type
C1.2E	C1	C1.2	EPC
C1.2Fb	C1	C1.2	Fb
C2.2E	C2	C2.2	EPC
C2.6Fb	C2	C2.6	Fb
C3.6E	C3	C3.6	EPC
C3.6Fb	C3	C3.6	Fb

Table 2: C samples established from the minor salivary glands of non-disease subjects.

Thereafter, the data taken from diseased patient group (S- group) and control patient (C-control) was analyzed to for up- regulation and down regulation of Gene expression from huge database of all the genes expressed in this group of patients. A group of gene expression that had showed high (positive) or low (negative) fold change in epithelial cells of patient versus the control were selected for further analysis. Another group of genes expressed that had showed high (positive) or low (negative) fold change in fibroblast were shortlisted to further analysis.

Quantitative Real Time-PCR:

Total RNA was isolated using Trizol reagent according to the manufacturer's instructions (Invitrogen) and 0.5ug total RNA was reverse transcribed using Protoscript M-MuLV First strand cDNA Synthesis Kit (New England Biolabs, Ipswich, MA). q-PCR was performed using the SYBR Green PCR Master Mix on an ABI Sept OnePlus machine according to the manufacturer's instructions (Invitrogen). Relative expression of each gene was determined using the Delta amplification cycles for Fold change and normalized to the housekeeping gene Gapdh. Primer sequences used in this study are listed in the Table 5. Statistical analysis was performed using Student T-test. In all the experiments in his study, $p < 0.05$ was considered statistically significant.

Immunohistochemistry:

Minor salivary glands from Sjogren's and non-disease patients were fixed in 10% buffered PBS and sent to the BUMC Histopathology Core Facility for sectioning. Slides were prepared for DAB Staining protocols as follows: First, the slides were rehydrated by incubating in 2 rounds of xylene for five min, and then incubated in 100%, 90%, and 75% ethanol for three min each and transferred to PBS.

Next, antigen retrieval was performed by incubating the slides in 10mM citrate buffer at 95°C for 20 min and cooled down for 20 min at room temperature.

Using a PAP pen, each section was delineated to create a hydrophobic boundary. The slides were then incubated in the blocking buffer (10% FBS in PBS) for 30 min, followed by incubation with anti-63 primary antibody (4A4 mouse monoclonal, Santa Cruz, Cat #sc-8431) diluted 1:20 in blocking buffer. The slides were kept overnight at 4°C. The slides were then washed in PBS for ten minutes, followed by incubation with the secondary antibody HRP-conjugated goat anti-mouse IgG (KPL, #474-1806) diluted 1:150 in PBS for 1hr. The slides were then washed for 10 mins in PBS. DAB stain was prepared and incubated for 5-20 min using the DAB staining kit (KPL, #54-10-00). Finally, the slides were washed in water for 10 min. Then the slides were dipped in hematoxylin solution for ten seconds and washed in water for five min to develop the color. Subsequently, the slides were dehydrated by incubation in 75%, 90%, and 100% ethanol for three min and then four rounds of incubation in xylene for three min each. The mounting was done using Permount solution (Fisher Scientific, #SP15-100) and cover glasses. The slides were monitored under a microscope, and images were taken with a panoramic scanner (3DHistech).

Immunofluorescence of cultured cells:

The cells in wells were rinsed with PBS and fixed for 10 min in 10% buffered formalin. Then the cells were permeabilized with 0.1% Triton in PBS (PBST) for 5 min and blocked in the blocking buffer (10% FBS in PBST), followed by incubation with primary antibodies at 4ºC overnight.

The primary antibodies and dilutions used were: anti-p63 (4A4 mouse monoclonal, Santa Cruz, Cat #sc-8431, 1:20); anti-cytokeratin 5 (rabbit polyclonal, Biolegend, #905501, 1:5000); and anti-pancytokeratin (rabbit polyclonal, Proteintech, #26411-1-AP, 1:200). At the end of the incubation period, the cells were washed three times in PBST for 2 min each.

Subsequently, the cells were incubated with secondary antibodies Alexa 594-goat anti-mouse IgG and Alexa 488-goat anti-rabbit IgG (Molecular Probes), diluted 1:1000 in PBST for 1 hr. The slides were covered with foil to avoid bleaching due to the exposure to light. The cells were then washed three times in PBST for 2 min each. At the end, the cells were incubated with Hoechst 33342 (Invitrogen) for 5 min and washed in PBS three times for 2 min each. Fluorescence images were taken using a high-resolution microscope (Keyence, BZ-X710).

Western Blotting:

Cell lysates were prepared by suspending the cultured cells in sample buffer and boiled for 10 min in a heat block. Two SDS-PAGE gels were prepared, and protein samples were run at 20A/gel for 1.5-2 hrs. The proteins were transferred to the membrane at 100 V in cold transfer buffer. The membranes were then rinsed in PBST and blocked overnight at 4°C in blocking buffer (5% v/w milk in PBST). The next day the blots were incubated with the primary antibody diluted in blocking buffer overnight at 4°C. The primary antibodies used were anti-p63 (4A4 mouse monoclonal, Santa Cruz, #sc-8431, 1:50); and anti-tubulin (12G10 mouse monoclonal, IOWA Hybridoma bank, #12G10, 1:100).

The blots were washed three times for 10 min each in washing buffer (PBST) with gentle agitation. Subsequently, the membranes were incubated with secondary antibody (HRP-conjugated Goat anti- mouse IgG, Company, #, 1:50000) in PBST for 1hr at room temperature. The membranes were then washed three times for 10 min each in washing buffer with gentle agitation. ECL solution (Amersham) was used to develop color reaction according to the manufacturer's directions. Blot signals were captured using the Bioluminescent Image Analyzer (Model: Image Quant LAS 4000, GE Healthcare).

RESULTS

[1] Expression of p63 in the minor salivary gland epithelia of patients with SS and non-disease controls. Senoo lab has recently made a novel observation that mice conditionally expressing the transcription factor p63 in epithelial progenitor cells of the salivary glands develop SS-like autoimmune disease. These observations indicate that abnormal activities of epithelial progenitor cells in the salivary glands can contribute to the pathogenesis of SS. In this study, we first asked whether expression of p63 is increased in the salivary gland epithelia in patients with SS compared to non-disease controls. We collected lip biopsies of minor salivary glands from patients with SS (n=3) and non-disease controls (n=3) at Tufts School of Dental Medicine (Boston) under the collaboration with Dr. Athena Papas (Tufts Dental). We also used paraffin blocks of minor salivary glands of patients with SS (n=55) and non-disease controls (n=10), previously collected at Tsukuba University School of Medicine (Ibaraki, Japan) by our collaborator Dr. Takayuki Sumida (Tsukuba Medical).

Staining of the sections with anti-p63 monoclonal antibodies show that expression of p63 is detected throughout the glands and enriched in the ductal epithelia in both patients with SS and non-disease controls (Figure 4).

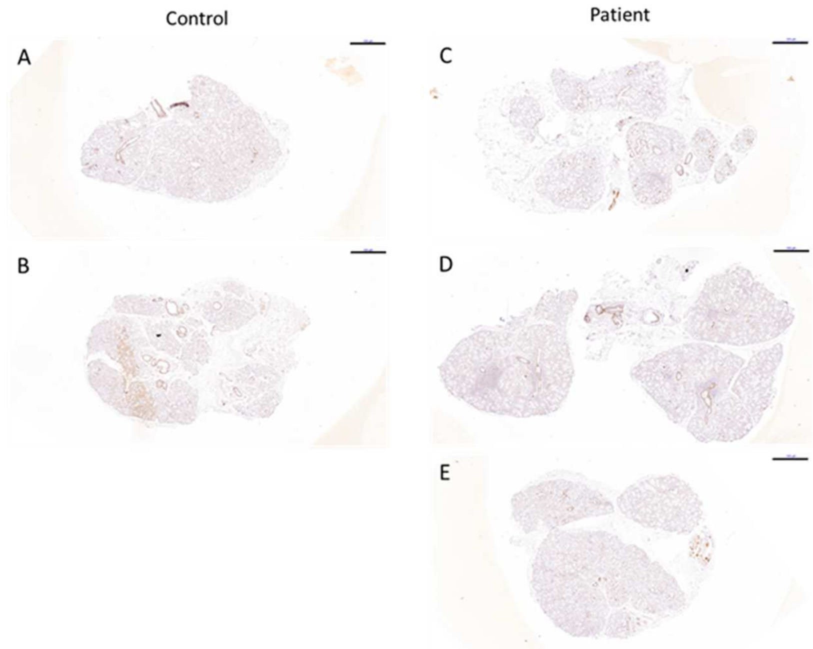

Figure 4: Expression of p63 in the minor salivary glands of patients with SS and non-disease controls (low magnification). Data shown are immunohistochemistry of paraffin sections of the minor salivary gland biopsies stained with anti-p63 antibodies (brown), followed by counterstaining with hematoxylin. A and B represent the control samples C3a and C3b, respectively, while C- E represent the SS samples S3a, S4a, and S5b, respectively. Scale bars=500µm.

At a higher magnification, it seems the frequency of p63-positive basal cells was higher in some ducts of patients with SS compared to that of non-disease controls (Figure 5). To determine the significance of these preliminary findings, we analyzed additional 65 cases including 55 SS and 10 non-disease control cases.

We categorized the ducts into the main excretory ducts (MED), intratubular excretory ducts (IED), and striated ducts (SD), and further subdivided into two groups: those that are associated and non-associated with infiltrated lymphocytes (Figure 6). Our data show that the frequency of p63-positive ductal epithelia was significantly higher in those with periductal infiltrates than those without in SS patients or corresponding normal ducts in non-disease controls (Figure 6). As this was true in all three duct systems, we conclude that increased expression of p63 in ductal epithelia associates with periductal inflammation in patients with SS.

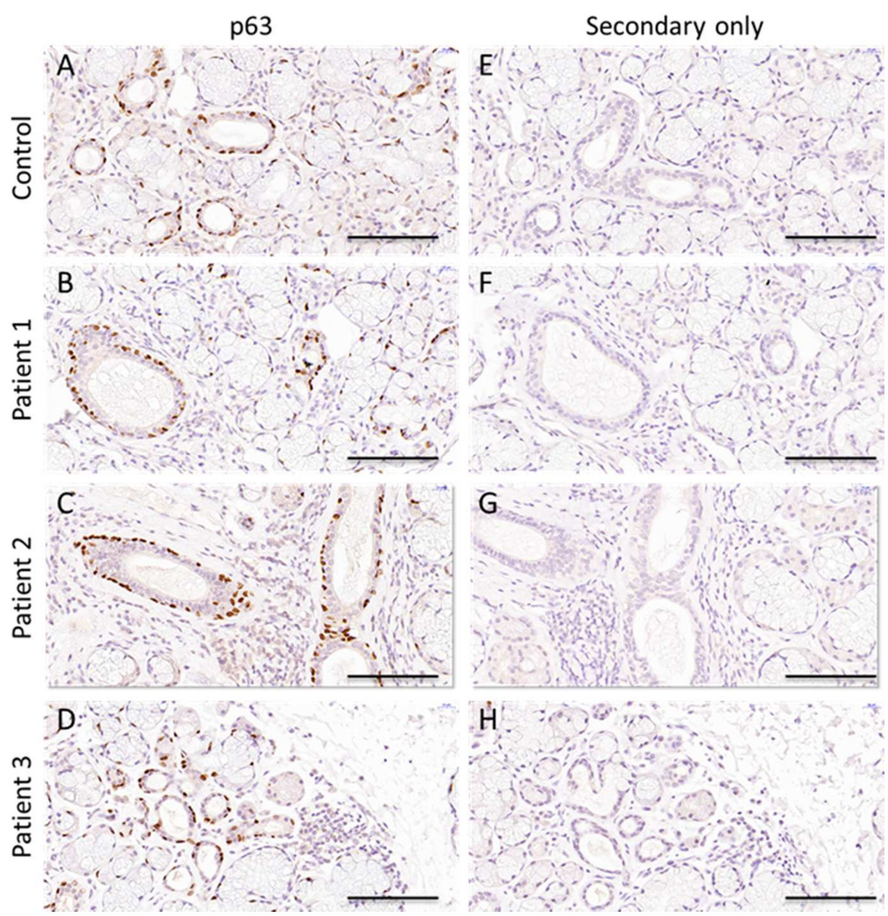

Figure 5: Expression of p63 in the minor salivary glands of patients with SS and non-disease controls (higher magnification). Shown are consecutive from Figure 4. The panels A-D represent the sample C3a, S3a, S4a, and S5b, respectively. The right panels E-H show corresponding negative staining. Scale bars=100μm.

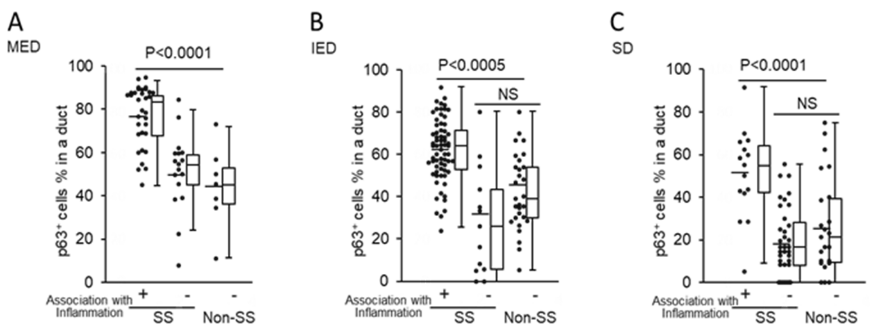

Figure 6: Increased p63+ cell frequency in the basal layer of inflamed ducts of the minor salivary glands of patients with SS. MED, main excretory duct; IED, interlobular excretory duct; SD, striated duct. "+" and "-"represent the presence or the absence of periductal inflammation, respectively. NS, not significant.

[2] Determination of relative p63 levels in ductal epithelial progenitor cells using primary culture of the minor salivary glands biopsied from patients with SS and non-disease controls.

Ductal epithelial progenitor cells derived from patients with SS and non-disease controls were grown in culture according to the method Senoo lab has recently established (29).

This method utilizes a combination of low calcium media and a small molecule inhibitor of the TGF signaling pathway and permits epithelial progenitor cells to grow without any obvious sign of differentiation. Indeed, as shown in Figure 7, both SS and control epithelial cells grown in culture show relatively small nuclear to cytoplasmic ratios, a hallmark of undifferentiated immature epithelial cells. In addition, epithelial cells in both cultures show high expression of p63 and cytokeratin 5, two major markers of epithelial stem/progenitor cells (Figure 8). To compare p63 levels between the SS samples and controls, we employed Western blot analysis of whole cell lysates of ductal epithelial progenitor cells as described above. Our results show that, although variable, p63 levels in epithelial progenitor cells derived from patients with SS are significantly higher than those from the control subjects (Figure 9). Together with the data shown above (see Figure 6), our results indicate that ductal epithelial progenitor cells in patients with SS express higher levels of p63 than those in non-disease controls, contributing to the increased frequency of p63-positive epithelial cells in the ducts of patients with SS.

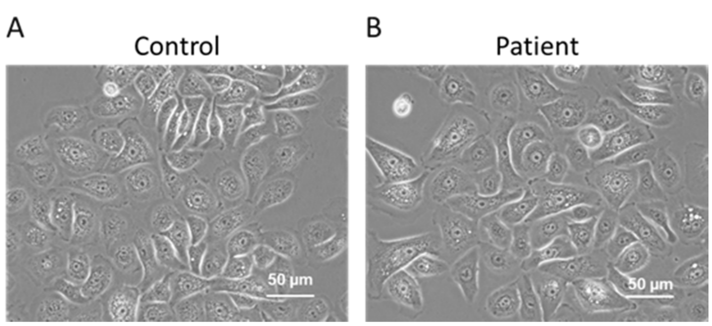

Figure 7: Brightfield images of primary ductal epithelial progenitor cells derived from the minor salivary glands of patients with SS and non-disease control.

A and B represent the samples C1.2 and S3.1, respectively. Note that both progenitor cells are morphologically similar and show small nuclear-to-cytoplasmic ratios, an indication that they are relatively immature. Scale bars=50μm.

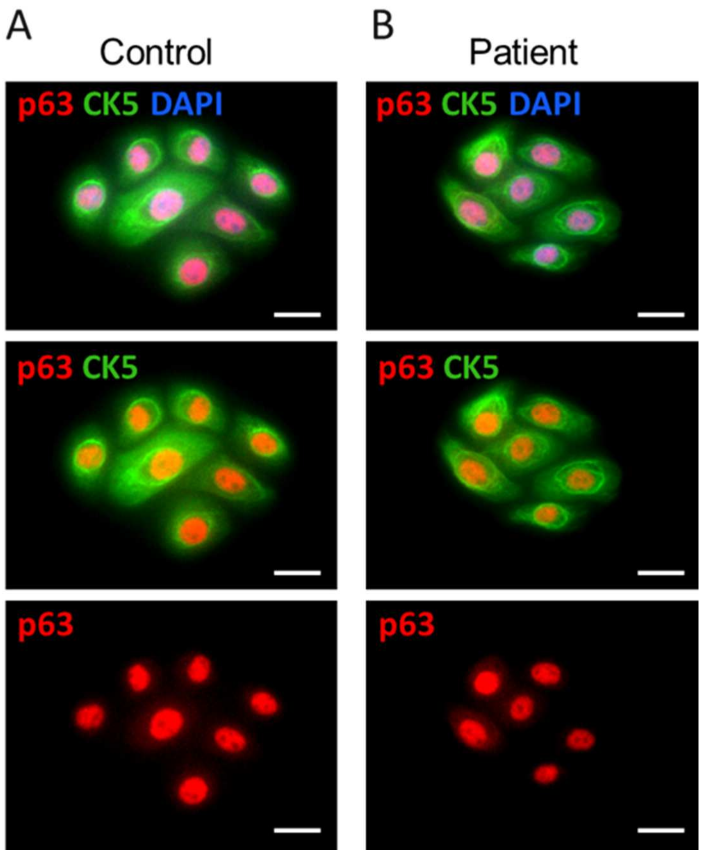

Figure 8: Expression of p63 and CK5 in primary ductal epithelial progenitor cells derived from the minor salivary glands of patients with SS and non-disease control. Data shown are representative immunofluorescence images of the cells stained with anti-p63 antibodies and anti-CK5 antibodies, counterstained with DAPI. Upper panels: merged images with p63 (red), CK5 (green), and DAPI (blue) staining, Middle panels: double staining for p63 and CK5, Lower panels: p63 staining only. Scale bars=10μm.

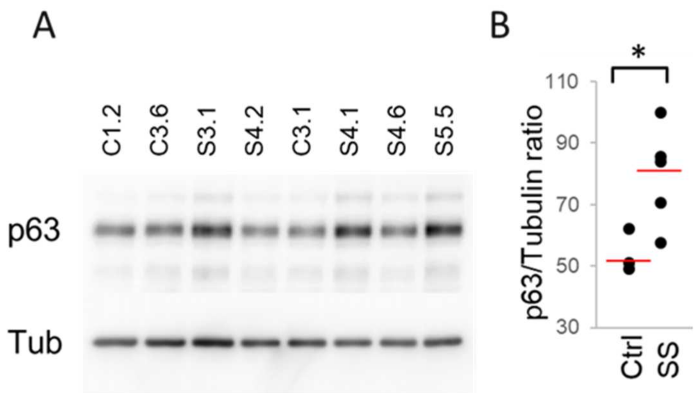

Figure 9: Quantification of p63 levels by Western blot analysis using lysates of primary epithelial progenitor cells. A. Expression of p63 in the labial salivary gland epithelial progenitor cells derived from patients with SS and non-disease controls. Tubulin was used as a loading control. B. Quantification of (A) showing the p63/Tubulin ratios in control versus SS samples. *, $p<0.01$.

[3] Identification of the potential effectors driving the pathogenesis of SS using deep-RNA sequencing of primary ductal epithelial progenitor cells of patients with SS and non-disease controls. The epithelial cells derived from patients with SS and non-disease controls were used to extract RNA for deep-RNA sequencing. The samples analyzed include ductal epithelial progenitor cells and case-matching mesenchyme (i.e., fibroblasts), both from patients with SS and non-disease controls. Principal Component Analysis shows that epithelial samples and fibroblast samples, as well as SS samples and C samples, were well separated in their gross gene expression profiles (Figure 10).

These results indicate that not only epithelial component but also the mesenchymal component show differential gene expression pattern between patients with SS and non-disease controls, an intriguing finding to be explored in future studies.

The genes selected with the cut off value of 2.0-fold change (a total of 218 genes) were used to create a heatmap data (Figure 11). Our results show that expression of three quarters of the selected genes was higher in patients with SS than in non-disease controls and that such a trend was epithelial-specific. To narrow down the candidate genes involved in the pathogenesis of SS, we cross-referenced the 218 genes identified in this study with the 6 major SS-associated datasets available in public (Table 3). This strategy has allowed us to identify 11 candidate genes (Figure 12), including the CD86 molecule known to be directly involved in the activation of immune cells in some autoimmune diseases. We validated the differential expression of each candidate gene by q-PCR using gene-specific primer sets (Figure 13).

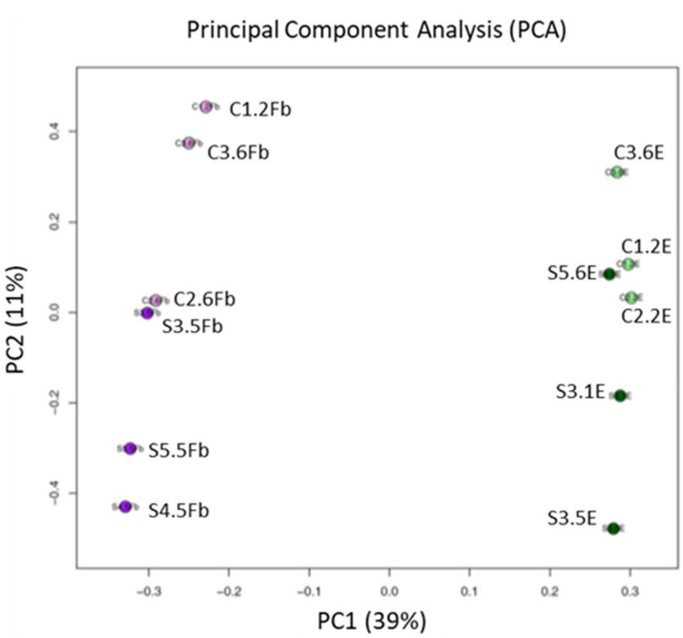

Figure 10: Principal Component Analysis (PCA) of the deep-RNA sequencing of ductal epithelial progenitor cells and case-matching fibroblasts. E and Fb represent epithelial progenitor cells and fibroblasts, respectively. C and SS indicate control subjects and patients with SS, respectively. Note that epithelial samples and fibroblast samples, as well as SS samples and C samples, were well separated in this PCA analysis.

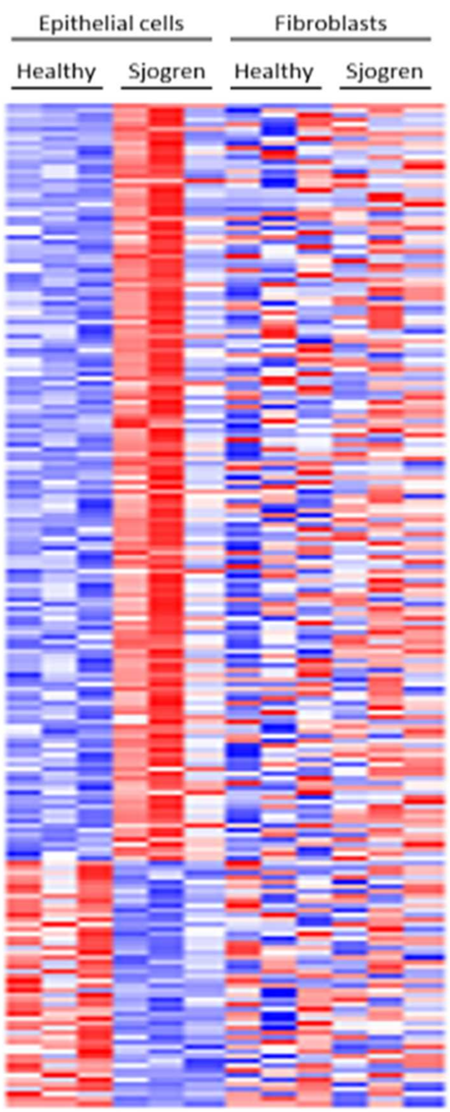

Figure 11: Heat map of gene expression in ductal epithelial progenitor cells and case-matching fibroblasts, both derived from labial biopsies. Red and blue indicate relatively high and low expression of each gene, respectively.

List	Description	No. of genes	Overlap with our dataset	Common genes
This study (Deep-RNA sequencing data)	Cultured human epithelial cells from minor salivary glands	218	11	AQP3(2.11),CD86(6.65), DU5P13(4.69), HLA-C(2.84) ,IFITM2(3.09),MDGA1 (6.65),MMP28(2.09),PRKCQ (4.35), RASGRP1(22.26),SPOCD12 (.23),TSHZ2(4.72)
Gottenberg 2005	Minor salivary glands were compared between 7 patients with SS and 7 controls	90	2	IFITM2(2.04), TSHZ2(2.86)
GSE127952	Minor salivary glands of 8 female patients with primary SS & healthy volunteers.	230	4	AQP3(-2.02),CD86 (2.04),DUSP13(-2.47),SPOCD1(-2.06)
GSE23117_GREENWELL	Whole minor salivary glands of patients with SS and control.	484	7	AQP3(-2.02),CD86(2.16) ,MDGA1(2.14), MMP28(2.07),RASGRP1 (2.06)

List	Description	No. of genes	Overlap with our dataset	Common genes
GSE40568_Tsuboi	Labial salivary glands of SS and healthy controls.	36	X	
GSE80805	Minor salivary glands from primary SS & non -SS patients.	45	X	
Hjelmervik 2005	Minor labial submucosal salivary glands.	76	1	HLA-C (-6.69)

Table 3: Extraction of the common genes between our deep-RNA sequencing data and SS-associated genes in literature. The table shows a list of datasets that were publicly available and used for searching for the common genes with our deep-RNA sequencing results.

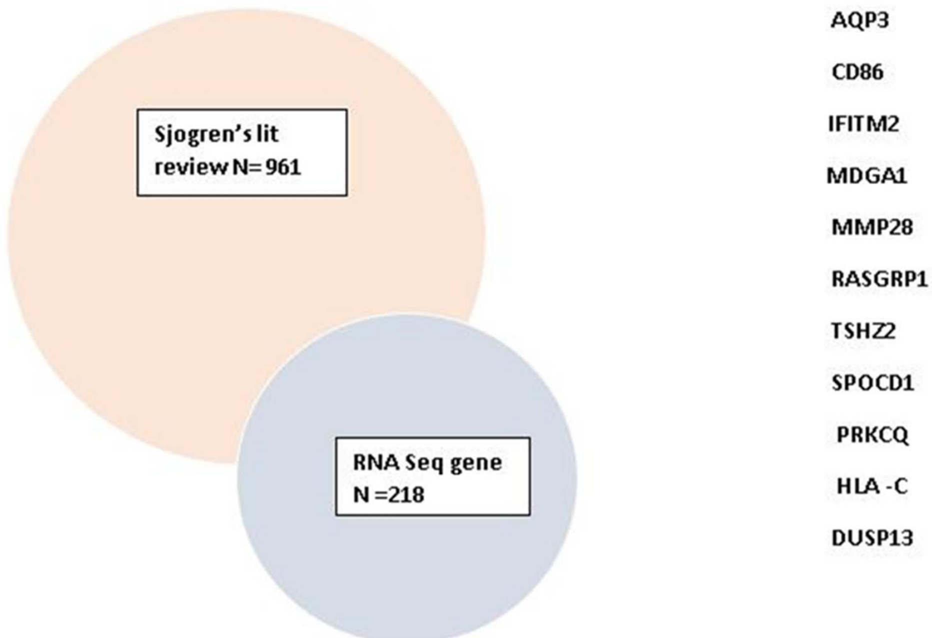

Figure 12: Diagrammatic representation of genes common in our deep-RNA sequencing data and publicly available SS-associated genes. The genes on the right constitute the common genes extracted in this study.

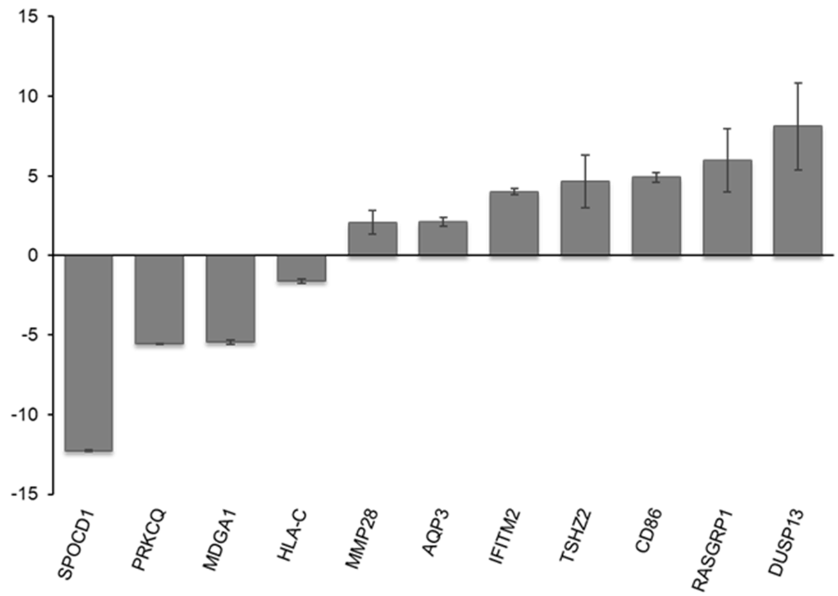

Figure 13: Validation of differential gene expression by q-PCR. Each individual gene was quantified by q-PCR using cDNA prepared from ductal epithelial progenitor cells derived from patients with SS and non-disease controls. Data shown are fold increase in the SS samples over the control subjects.

Genes	q-PCR	Deep-RNA seq
SPOCD1	-12.28	-2.23
PRKCQ	-5.56	-4.35
MDGA1	-5.45	-6.65
HLA-C	-1.60	-2.84
MMP28	2.08	2.09
AQP3	2.14	2.11
IFITM2	4.01	3.09
TSHZ2	4.63	4.72
CD86	4.92	6.65
RASGRP1	6.00	22.62
DUSP13	8.10	4.69
TP63	1.16	1.10

Table 4: Comparison of the fold change of gene expression in deep-RNA sequencing and q-PCR. Fold changes in gene expression as determined by deep-RNA sequencing and qPCR are summarized for each gene. Note: The TP63 gene was included as a reference.

CONCLUSION:

We have shown that epithelial progenitor cells of the salivary glands in patients with SS express higher levels of p63 than those in non-disease controls. In addition, we identified 11 potential effectors that drive the pathogenesis of SS under the influence of the p63 expression.

DISCUSSION

In this study, we have shown that epithelial progenitor cells of the salivary glands in patients with SS express higher levels of p63 than those in non-disease controls, potentially contributing to the altered gene expression in SS. Interestingly, however, our q-PCR result shows that the p63 transcription was similar between ductal epithelial progenitor cells of patients with SS and those in non-disease controls. This finding raises an intriguing possibility that the alterations of the mechanisms that lead to the increased stability of p63 play a key role in the development of SS. Identification of the mechanisms that control the p63 stability in ductal epithelial progenitor cells will open up a new window for developing novel therapeutic options to treat SS.

From this study there are eleven shortlisted genes, that could be influencing the pathway to SS. CD86 and MMP28 are particularly interesting because of their roles in the autoimmune disease processes. CD86 is a gene that encodes a type I membrane protein that is a member of the immunoglobulin superfamily. This protein is expressed by antigen-presenting cells, and it is the ligand for two proteins at the cell surface of T cells, CD28 antigen and cytotoxic T-lymphocyte-associated protein 4. Binding of this protein with CD28 antigen generates a costimulatory signal for activation of T-cells. Therefore, this genes upregulation may increase the inflammatory response and immune response. Future direction includes exploring this individual gene contribution (8).

Another important factor we identified is MMP28, a proteins of the matrix metalloproteinase (MMP) family. They are involved in the breakdown of extracellular matrix for both normal physiological processes, and disease processes. This gene encodes a secreted enzyme that degrades casein. Its expression pattern suggests that it plays a role in tissue homeostasis and in wound repair. MMP overexpression can lead to tissue destruction which is characteristic of chronic inflammatory diseases such as rheumatoid arthritis and scleritis (1).

Future studies should focus on the functional analysis of each p63-assocciated candidate gene in the pathogenesis of SS.

APPENDIX:

Gene Name		Sequence
AQP3	Forward	GGGGAGATGCTCCACATCC
	Reverse	AAAGGCCAGGTTGATGGTGAG
CD86	Forward	CTGCTCATCTATACACGGTTACC
	Reverse	GGAAACGTCGTACAGTTCTGTG
DUSP13	Forward	AGCCGTGTGGACGAAGTTTG
	Reverse	CTGCCGTAGAAGTCAGGC
HLA-C	Forward	CCATGAGGTATTTGTGGACCG
	Reverse	TCTCGGACTCTCGTCGTCG
IFITM2	Forward	ATGAACCACATTGTGCAAACCT
	Reverse	CGGAGTACGGCGAATGCTATGAA
MDGA	Forward	TGGACCAAGACGGCAGGTA
	Reverse	CGTGCAATACGCTCGATGC
MMP28	Forward	TACCAACAGTTATGCGGCCTG
	Reverse	CAAAGCGTTTCTTACGCCTCA
PRKCQ	Forward	ATGTCGCCATTTCTTCGATT
	Reverse	ACATACTTCTTTGACGAGCACAG
SPOCD1	Forward	GTTGTCCGTTCCATGCAGGA
	Reverse	ATTGGTGCCTTGTGTCAGGTC
RASGRP1	Forward	TGGAAACCTGTGTCGAAGTAAC
	Reverse	ACTCCTCCATAGTGTCTGTCAAG
TP63	Forward	GGACCAGCAGATTCAGAACGG
	Reverse	AGGACACGTCGAAACTGTGC
GAPDH	Forward	AGCCACATCGCTCAGACAC
	Reverse	GCCCAATACGACCAAATCC

Table 5 :List of primers.

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CURRICULUM VITAE:

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