Salivary Gland Stem Cells Age Prematurely in Primary Sjögren's syndrome

Authors: Sarah Pringle[1]* PhD, Xiaoyan Wang[1] MSc, Gwenny M.P.J. Verstappen[1] PharmD PhD, Janneke H. Terpstra[1] BSc, Clarence K. Zhang[2] PhD, Aiqing He[3] PhD, Vishal Patel[3] PhD, Rhiannon E. Jones[4] BSc, Duncan M. Baird[4] PhD, Fred K. L. Spijkervet[5] DDS MD PhD, Arjan Vissink[5]DDS MD PhD, Hendrika Bootsma[1] MD PhD, Robert P. Coppes[67†] PhD. and Frans G.M. Kroese PhD. [1†]

Affiliations:

[1]Department of Rheumatology and Clinical Immunology, University of Groningen, University Medical Center Groningen.[2]Translational Bioinformatics, Bristol-Myers Squibb, 3551 Lawrenceville Princeton, Lawrence Township, NJ 08648, USA.[3]Integrated genomics, Bristol Myers Squibb, 311 Pennington-Rocky Hill Rd., Pennington, NJ 085034, USA.[4]Division of Cancer and Genetics, Cardiff University School of Medicine. [5]Department of Oral and Maxillofacial Surgery, University of Groningen, University Medical Center Groningen.

[6]Department of Cell Biology, University Medical Center Groningen. [7]Department of Radiotherapy, University of Groningen, University Medical Center Groningen.

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***Corresponding author:** Telephone: +31503616604; Email: s.a.pringle@umcg.nl

† These authors contributed equally to this work

Abstract

Objective: A major characteristic of the autoimmune disease primary Sjögren's syndrome (pSS) is salivary gland (SG) hypofunction. Resident salivary gland stem cell (SGSC) inability to maintain homeostasis and saliva production has never been explained, and limits our comprehension of mechanisms underpinning pSS.

Methods: SGSCs were isolated from parotid biopsies of controls and patients classified as pSS or incomplete pSS, according to ACR-EULAR criteria. Self-renewal and differentiation assays determined SGSC regenerative potential, RNA was extracted for RNASeq analysis, STELA analysis employed to determine telomere length, and frozen tissue used for immunohistochemical analysis.

Results: Here we show that SGSCs isolated from pSS parotid gland biopsies are regeneratively inferior to healthy controls. We demonstrate that SGSCs from pSS biopsies are not only lower in number and less able to differentiate, but are likely to be senescent, as revealed by telomere length analysis, RNASeq and immunostaining. We further report that SGSCs exposed to pSS-associated proinflammatory cytokines are induced to proliferate, express senescence associated genes, and subsequently differentiate into intercalated duct cells. We also localize $p16^{+}$ senescent cells to the intercalated ducts in pSS SG tissue, suggesting a block in SGSC differentiation into acinar cells.

Conclusion: This study represents the first characterization of SGSCs in pSS, and also the first linkage between an autoimmune disease and a parenchymal premature ageing phenotype. The knowledge garnered in this study argues that disease modifying anti-rheumatic drugs used to treat pSS are not likely to restore saliva production, but should be supplemented with fresh SGSCs to recover saliva production.

Between 0.4 and 3.1 million people in the U.S. suffer from the autoimmune disease primary Sjögren's syndrome (pSS) 1. Presenting clinically in predominantly women (9:1 ratio), pSS is a multi-faceted syndrome most often associated with production of autoantibodies (SSA/Ro, SSB/La), infiltration of the salivary glands with lymphocytes, and hyposalivation (reduced secretory function of the salivary glands). Other symptoms may include neurological aspects, lung complaints and chronic fatigue. Lymphocytic infiltration of salivary glands is characterized and measured clinically by presence of immune foci, gatherings of more than 50 lymphocytes in the salivary glands, associated with the striated ducts. The periductal infiltrates may evolve into ectopic lymphoid tissue harboring germinal centers (sites of memory B cell formation). In addition, the relative number of IgA plasma cells decreases, in parallel with glandular dominance of IgG producing plasma cells. Mucosa-associated lymphoid tissue (MALT) lymphomas are also frequently observed in salivary gland tissue of pSS patients. All these aspects reflect the B-cell dominated phenotype of pSS 2. Given these characteristic lymphocytic infiltrates, the logical conclusion is that lymphocytic infiltration of salivary glands is the causative factor underpinning hyposalivation. More recent, detailed research however has clearly demonstrated to the contrary that the correlation between salivary flow and degree of inflammation is poor 3–11.

In healthy SGs, homeostasis is maintained by proliferation and differentiation of tissue resident salivary gland stem cells (SGSCs). According to one of the prevailing views in the field these cells reside in striated ducts, from where they differentiate first towards intercalated ducts and subsequently to acinar cells 12–16. Other groups have identified progenitor cell populations within the acinar cell subset, and alternatively also suggested that acinar cells themselves are capable of

maintaining SG homeostasis through self-replication 17,18. Regardless of whichever niche you consider SGSCs to reside in, the apparent lack of ability of SGSCs to maintain SG homeostasis in pSS has never been explained, and likely contributes to hyposalivation development in pSS. Here we employ our recently developed protocols for SGSC isolation to probe involvement of SGSCs in pSS 12–14,16,19. We demonstrate that SGSCs in pSS are likely to be senescent, a phenotype that may be induced by exposure to pSS-associated pro-inflammatory cytokines.

Materials and Methods

Source of salivary gland tissue

For healthy control biopsies, parotid SG tissue was obtained from donors (after informed consent and IRB approval (METC2016/010) who were treated for a squamous cell carcinoma of the oral cavity. In these patients an elective head and neck dissection procedure was performed. During this procedure parotid SG is exposed and removed as part of the dissection procedure. This tissue does not contain malignant cells, as oral squamous cell carcinoma does not disseminate to the parotid salivary gland. For biopsies from pSS and incomplete pSS patients, biopsies were taken during routine biopsy for pSS diagnosis work-up trajectory. Patients were classified as pSS if fulfilling the 2016 ACR-EULAR criteria for pSS 20. Incomplete pSS patients did not fulfill these criteria, were not taking hyposalivation-inducing medication, but did demonstrate either objective symptoms of dry mouth or SSA autoantibody presence. All patients gave IRB consent and approval (METc 2016/010).

Stem cell isolation

Parotid SG biopsies were harvested from oral squamous cell cancer patients with healthy parotid gland tissue , incomplete pSS patients and pSS patients were collected after surgery into Hank's Balanced Salt Solution (HBSS) containing 1 % bovine serum albumin (BSA; Invitrogen). Biopsies

were mechanically digested using the gentleMACS dissociator (Miltenyi Biotec) or manually with scissors, and simultaneously subjected to digestion in HBSS/1% BSA buffer containing 0.63 mg/mL collagenase II (Invitrogen) and 0.5 mg/mL hyaluronidase (Sigma Aldrich), and calcium chloride at a final concentration of 6.25 mM, for 30 minutes at 37 ˚C. Forty mg of tissue was processed per 1 mL buffer volume, total volume was adjusted according to biopsy weight. Digested cells were collected by centrifugation, washed twice in HBSS/1 % BSA solution, and passed through 100 µm cell strainers (BD Biosciences). Resultant cell suspensions were collected again by centrifugation and resuspended in SGSC medium consisting of 40 % Dulbecco's modified Eagle's medium:F12 medium, Pen/Strep antibiotics (Invitrogen), Glutamax (Invitrogen), 50 % Wnt3a conditioned medium, 10 % R-spondin conditioned medium (derived from the RSPO-1 cell line, Amsbio), 20 ng/mL epidermal growth factor (EGF) (Sigma Aldrich) 20 ng/mL fibroblast growth factor-2 (FGF2) (Sigma Aldrich), N2 (Invitrogen), 10 mg/mL insulin (Sigma Aldrich), and 1 mM dexamethasone (Sigma Aldrich), 10 µM Rho Kinase Inhibitor (Abcam), 5 µM TGFß inhibitor (A8301, ToCris Bioscience) and 12.5 ng/mL Noggin (Peprotech). 800,000 of primary isolate cells were resuspended in 25 µL of SGSC medium, combined with 50 µL of Basement Membrane Matrigel (BD Biosciences) and deposited in center of 12-well tissue culture plates. After letting the gels solidify (20 minutes at 37 ˚C), 1 mL of stem cell medium was added per well. After 3-5 days of culture, primary spheres formed were released from Matrigel by incubation in 1 mg/mL Dispase (1 hour at 37 ˚C; Sigma). Primary spheres of a minimum size of 50 µM were counted and used to establish primary sphere yield per mg of biopsy material. To correct primary sphere yield for the site of biopsy, HC and pSS primary sphere yields were multiplied by factors of 4.1 and 11.95 respectively. Multiplication factors were derived from yield of primary spheres isolated from the SGSC-rich area, according to van Luijk et al 21.

Cytospot preparation and quantification

100 uL of cell suspension obtained after SGSC isolation protocol was added into cytospin funnel, after pre wetting of coated microscope slides with 1 % BSA/PBS solution. After centrifugation at 300 rpm for 2 minutes, slides were air dried, and fixed with 4 % PFA at room temperature for 20 minutes. Haematoxylin and eosin staining was then performed as per standard protocols. Number of acinar and ductal cells was determined by capturing images of 3 areas of the cytospot per sample. Total cell number in each areas was determined by counting hematoxylin stained nuclei. Acinar cells were identified by characteristic triangular morphology and predominant hematoxylin staining. Ductal cells were identified by heavily eosin stained cytoplasm. Proportion of each cell type was expressed as percentage of total cells. For CD45+ quantification, cytospots were fixed as above, then permeabilized in 100 % ethanol (20 minutes at -20 ˚C), washed in PBS and then incubated in mouse anti CD45 antibody (DAKO: 1 hour at RT) diluted 1:100 in 1 % BSA 0.05/Tween/PBS. Following PBS washing, goat anti-mouse secondary antibody conjugated to Alexaflour-488 was added onto cytospots at 1:300 dilution in 1 % BSA / 0.05 Tween / PBS, and incubated at room temperature for 1 hour. Following final PBS washes, nuclei were counterstained with DAPI and cytospots visualized using the a Leica 6000 Series microscope.

Flow cytometry and FACS of salivary gland isolate

Cell suspension post-isolation where appropriate were dispersed to single cells. Cells were immunolabeled with antibodies against the following human proteins, conjugated to fluorophores as indicated: EpCAM-eFlour660 (eBioscience, 1:20), CD45-PE-Cy5 (Biolegend, 1:50), CD19-BUV737 (eBioscience, 1:50), CD3-APC-eF700 (eBioscience, 1:50), CD56-PE-Cy7 (Biolegend, 1:50), CD4-APC-eF780 (eBioscience, 1:50), CD24-PE-Cy7 (Biolegend, 1:20), Ki67-FITC (Thermofisher Scientific, 1:200). For intranuclear staining for Ki67, the eBioscience Foxp3

Transcription Factor Buffer Set was used, as per manufacturer's instructions. Staining for K14 and SMA was performed in two steps using rabbit anti human K14 (Abcam, 1:100) and mouse anti human SMA (DAKO, 1:100) and Alexaflour-647-conugated secondary antibody (1:300). Antibodies were added in total volume of 100 µL 0.5 % BSA/PBS with 2mM EDTA (staining buffer), containing a maximum of 1 million cells. Staining was performed for 20 minutes on ice. Cells were collected by centrifugation and resuspended staining buffer for analysis with the LSR-II flow cytometer (BD Biosciences). Live-dead discrimination was performed using 80 ng / mL propidium iodide (Thermofisher). For FACS sorting of EpCAM+ cells from salivary gland isolate, staining was performed as above, with addition of 0.1 M Magnesium Sulphate (Sigma) and 50 µg / mL DNase (Sigma) into cell suspension to prevent cell clumping. Collected $CD45^+$ were harvested into stem cell medium collected by centrifugation and plated into Matrigel as described above. Gating strategy for flow cytometric analysis and FACS is shown in Supplementary Fig. 5.

Self-renewal

Following release of primary spheres from Matrigel as above, cells were dispersed to form single cell suspensions using 0.05 % trypsin-EDTA (Invitrogen), enumerated, and concentration adjusted to 0.4 x 10^6 cells per mL in SGSC medium. 25 µL of this cell solution was combined with 50 µL volumes of Basement Membrane Matrigel and deposited in the center of 12-well tissue culture plates. After solidifying the Matrigel for 20 minutes at 37 ˚C, gels were covered in stem cell medium as defined above. Organoids appeared 2–3 days post-seeding of single cells in Matrigel. Ten days after seeding, Matrigel was dissolved by incubation with Dispase enzyme as above. Organoids over 50 µM in diameter were enumerated, cells were processed to a single cell suspension using 0.05 % trypsin-EDTA and cell number determined. These data were used to

generate the organoid formation efficiency and population doublings. Population doublings (pds) were calculated according to the following formula:

$$pds = \frac{\ln 2 \,(\text{harvested cells}/\text{seeded cells})}{\ln 2}$$

Encapsulation in Matrigel was repeated to generate the next passage. This cycle was repeated four times (4 passages). At each the end of each passage an image was captured of the cells, using the Olympus CKX53 microscope and DP2-SAL software.

Mature organoid formation assay

For mature organoid formation assays, organoid cultures were supplemented with 1μM Isoproterenol was added per gel. Mature organoid formation was monitored over a two week period.

RNASeq

Total RNA was extracted from stem cells by using Absolutely RNA Miniprep kit (Agilent Technologies, CA. Cat: 400800) followed the manufacturer's recommendations. The integrity of RNA were examined by Agilent 2100 bioanalyzer. Subsequent sequencing was performed by using SMART-Seq v4 Ultra Low Input RNA Kit (Clontech, Cat: 634890) and Nextera XT DNA Library Prep Kit (Illumine, Cat: FC-131-1096) followed manufacturer's recommendations, prepared DNA libraries were sequenced on HiSeq 2500 System. Data quality assessment was performed to understand main source of variability, differential expression analysis and visualization were performed in R (packages PVCA, EdgeR and PHeatmap). Metacore pathway database was used for pathway enrichment analysis

Cytokine incubations with SGSCs

Cytokines were purchased as follows: human IL-6 (Peprotech; 200-06A) human IFNα (R&D Systems; 11100-1), and human TNFα (Peprotech; 300-01A) and reconstituted according to

manufacturer's directions. Dilutions for co-culture with cytokines were performed in such a manner that volume of cytokine added to medium was always 1 % of total medium volume. Medium was refreshed 2 times within the passage (days 3 and 6), in parallel with control cultures.

Whole mount and tissue immunocytochemistry

Mature organoids were released from Matrigel using Dispase, collected in round-bottomed 96-well plates, and fixed in 2 % PFA for 10 minutes. Frozen tissue sections were cut at a thickness of 8 µM, and fixed in 2 % PFA for 5 minutes. Staining was performed for all samples from this point following instructions of the Tyramide Signal amplification kit (Thermofisher). After hydrogen peroxide blocking and general blocking, primary antibodies were incubated with organoids, mature organoids or tissue sections overnight in PBS at 4 degrees. Dilutions of primary antibodies used for immunostaining were: rabbit anti-human amylase (1:100, Sigma A2863); rabbit anti-human aquaporin-5 (AQP-5), rabbit anti-human EpCAM (antibody as FACS analysis), mouse anti human IL6R (1:100; Thermofisher clone B-R6), mouse anti human TNFR1 (Thermofisher clone H398), rabbit anti human IFNαR (1:100; Abcam 62693), mouse anti-human p16 (1:100; Abcam 54210) and mouse anti human smooth muscle actin (1:100, Dako M0851). Nuclear counterstaining was performed with Hoechst 33342, at 1:300 dilution from 10 mg / mL stock solution, for 10 minutes at room temperature. Immunostainings were visualized using the Leica TCS SP8 confocal laser scanning microscope and Leica Application Suite software.

Telomere analysis

DNA was extracted from human salivary gland stem cells using a QIAmp DNA Micro Kit (Qiagen). Single telomere length analysis (STELA) was carried out at the XpYp telomere as described previously (Capper et al. 2007). Briefly 1 µM of the Telorette2 linker was added to 10 ng of purified genomic DNA in a final volume of 40 µL per sample. Multiple PCRs were

performed for each test DNA in 10 µL volumes incorporating 250 pg of DNA, 0.5 µM of the telomere-adjacent and Teltail primers, 75 mM Tris-HCl pH8.8, 20 mM $(NH_4)_2SO_4$, 0.01% Tween-20, 1.5 mM $MgCl_2$, and 0.5 U of a 10:1 mixture of Taq (ABGene) and Pwo polymerase (Roche Molecular Biochemicals). The reactions were processed in a Tetrad2 Thermal Cycler (BioRad). DNA fragments were resolved by 0.5 % TAE agarose gel electrophoresis and identified by Southern hybridization with a random-primed a-^{33}P-labeled (PerkinElmer) TTAGGG repeat probe, together with probes specific for the 1 kb (Stratagene) and 2.5 kb (BioRad) molecular weight markers. Hybridized fragments were detected using a Typhoon FLA 9500 Phosphorimager (GE Healthcare). The molecular weights of the DNA fragments were calculated using a Phoretix 1D Quantifier (Nonlinear Dynamics).

qPCR

Total RNA as was extracted from cultured cells as appropriate using the RNeasy Microkit (Qiagen), including DNase incubation, as per manufacturer's instructions. One µg of total RNA was reverse transcribed to cDNA using 0.5 µg oligo$(dT)_{15-18}$ primers, 1.0 mM dNTPs, 1X Reaction Buffer, 20U Ribolock and 200 U of RevertAid Reverse Transcriptase (all Thermo Fischer Scientific), in a total volume of 20 µL per reaction. cDNA product was diluted ten-fold in water and used at this concentration for qPCR. qPCR was performed using SsoAdvanced Universal SYBR Green qPCR Mastermix (Biorad), with primers at a final concentration of 500 nM from a 10 µM stock. 2.5 µL of diluted cDNA was used per reaction, and all reactions were performed in triplicate, in a total volume of 10 µL. Primer sequences can be found in Table S1. A 2-step qPCR cycle with the BioRad iCycler qPCR machine was used for target amplification according to SSoAdvanced Universal SYBR Green Mastermix instructions, and CFX Manager software for analysis.

Results

Salivary gland stem cells from pSS patients show reduced regenerative potential

We began by isolating SGSCs from parotid SG biopsies of control patients with healthy SGs (HCs) and pSS patients fulfilling the ACR-EULAR classification criteria 20. SGSCs are initially cultured from processed biopsies as primary spheres, in Wnt-containing medium. Three to five days later, spheres are dispersed to single SGSCs, and expanded in a 'self-renewal assay'. The cell suspension generated by the isolation process from pSS biopsies contained significantly less epithelial cells than HC biopsies and significantly more $CD45^+$ leukocytes, based on cell morphology and immunostaining on cytospots (Supplementary Figure 1, Supplementary Figure 2A-C). As previously reported for minor SGs, we detected a high proportion of B-cells and a predominance of $CD4^+$ T-cells within the flow-cytometry-defined $CD45^+$ fraction of the biopsy isolate (Supplementary Figure 2D,E) 22. SGSCs are epithelial cell adhesion molecule $(EpCAM)^{hi}$ in nature. The number of both spheres generated per $EpCAM^{hi}$ cell and yield of spheres per mg of biopsy was significantly lower (10 fold difference) from biopsies from pSS patients compared to HCs (Figure 1A,B, Supplementary Figure 2F). Data are presented normalized to mg of tissue to take account of the larger healthy salivary gland biopsies obtained. Primary sphere yield was not correlated with focus score (lymphocytic infiltration), supporting studies suggesting infiltration does not determine SG function (Supplementary Figure 2G).

We have previously demonstrated that SGSC yield decreases with age and that more SGSCs are present closest to the facial nerve in the parotid gland 21,23. Neither donor age or biopsy site was responsible for the decreased yield of SGSCs from pSS biopsies (Supplementary Figure 2H,I).

Stem cells are classically defined by their ability to proliferate and differentiate. When SGSCs from pSS biopsies were cultured as organoids to assess their proliferation capacity, we observed a significantly (up to 5-fold) lower self-renewal capability compared to HCs (Figure 1C-E)13. FACS selection of $EpCAM^{hi}$ cells from pSS biopsies, thus after removal of infiltrating leukocytes, did not rescue self-renewal potential of pSS-SGSCs, indicating that sole presence of $CD45^{+}$ cells in SGs of pSS patients is not responsible for the regenerative deficits observed (Supplementary Figure 3). Healthy SGSCs can be induced to proliferate and differentiate from organoids into α-amylase expressing mature organoids (Figure 1F,G). The lack of proliferative capabilities of SGSCs from pSS biopsies is reflected also in their greatly diminished ability to form mature organoids (Figure 1H). These data imply that the relatively few SGSCs present in pSS SGs harbor also defects in differentiation ability.

Salivary gland stem cells in pSS undergo extensive proliferation

In order to elucidate early events in SG pathology development in pSS which are not influenced by mass lymphocytic infiltration, we focused on patients classified as 'incomplete pSS' patients. These patients have some hallmarks of pSS (outlined in Supplementary Table 2) but with no positive lymphocyte focus score. This patient cohort was further not taking medication known to cause dry mouth complaints, all registered complaints of dry eyes and mouth associated with pSS development and did not fulfil the ACR-EULAR criteria. We consider these features indicative of early SG pathology development in pSS. When SGSCs were isolated from these patients, we observed that primary sphere yield from a small proportion (2/10) of these biopsies was markedly greater than the median of the HCs (Figure 2A). The yield of primary spheres from the remaining biopsies was comparable with pSS biopsy yield already demonstrated. Reasoning that the 2/10 patients with high yield represent an earlier disease stage24, we theorized that SGSCs receive

mitotic stimuli early in pSS. We performed RNASeq on organoids cultured from patients with incomplete pSS to investigate early events in pSS, and observed a cohort of 101 significantly upregulated genes in SGSCs from biopsy negative patients compared to HCs ($p<0.01$, Log10 fold change ≥ 2, Figure 2B).

When examined further, 18 of these genes were involved in cell cycle progression (both its promotion and inhibition) and DNA replication (Figure 2C, Figure S4). As shown in Figure 2C and Figure 2D, the β-galactosidase-like gene GLB1L2 was also significantly upregulated. B-galactosidase expression is associated with cellular senescence and ageing. Hypothesizing that SGSCs in pSS disease progression become senescent, we examined the telomere lengths of organoids cultured from pSS patients with positive SG biopsy evaluations (i.e. with lymphocytic infiltration), representing a later phase of pSS in terms of SG pathology. STELA analysis of telomere length revealed short telomeres of less than 4.5kb in length, in SGSCs from biopsy positive pSS patients (Figure 2E,F, clinical characteristics in Supplementary Table 2). Mean length of the lowest 10% of telomeres in HC SGSCs was significantly greater (4.80kb) compared to pSS SGSCs (1.59kb), suggesting that pSS SGSCs have a more extensive replicative history (Figure 2G). Mean ages of HC and pSS SGSC donors from which telomere analysis was performed were 77.3 and 61.5 years respectively, confirming that telomere difference was not due to advanced age of pSS SGSC donors.

Proinflammatory cytokines include proliferation and differentiation of healthy salivary gland stem cells

pSS is an autoimmune disease associated with glandular presence of classic proinflammatory cytokines, as exemplified by IFNα, TNFα and IL-622. Pro-inflammatory cytokines within the glandular tissue could provide mitotic signals driving SGSC exhaustion in pSS, leading to a

senescent ageing-like phenotype and ultimately hyposalivation. Considering the low yield of SGSCs from pSS patients, and in order to model the earliest phases of pSS, we employed HC SGSC cultures to investigate this hypothesis. qPCR and immunostaining of HC SGSC organoids at passage 2 demonstrated that HC SGSCs express receptors for the proinflammatory cytokines IFNα, TNFα and IL6 (Supplementary Figure 5, primers in Supplementary Table 1). When HC SGSCs were incubated from passages 1-4 with a cocktail of pro-inflammatory cytokines at concentrations matching those found in pSS patients' serum (IFNα 500 pg/mL; TNFα 40 pg/mL; IL-6 30 pg/mL) 25, we observed initially a significant increase in organoid formation efficiency, followed by a decrease to significantly below the levels of control cells (Figure 3A,B). Incubation with single cytokines did not induce significant proliferative effects, even at higher doses (Supplementary Figure 6). At passage 1 following cytokine exposure, expression of genes promoting cell cycle progression (CDK4, CDK6, CDC20), inhibiting cell cycle (E2F1, CDKN2D) and promoting senescence (p16 and p21) were upregulated (Figure 3C). Through definition of SGSC subsets using cell surface markers and costaining with the proliferation marker Ki67 (Supplementary Figure 7a-b), we also show that SGSCs resident in the basal layer of striated ducts (BSD cells) are responsible for the proliferation observed (Supplementary Figure 7c). We also suggest that proinflammatory cytokines induce differentiation of basal striated duct cells into intercalated ducts (ID cells) (Supplementary Figure 7c,d). Finally, p16 immunostaining was performed on sections of SG tissue in order to determine where senescent cells were located *in situ*. $p16^{+}$cells were found mostly in intercalated ducts in incomplete and complete pSS tissue. In contrast, $p16^{+}$ cells in HC SGs were found dispersed through the tissue, illustrating their full differentiation potential (Figure 3D-G).

Discussion

The origins of hyposalivation development in pSS have never been fully elucidated, although many studies have now firmly established that its development cannot be fully explained by extent of lymphocytic infiltration 3–9. Using salivary gland stem cells as tool to probe SG dysfunction in pSS, we show here that parotid gland biopsies of pSS patients contain fewer SGSCs, with reduced proliferation, differentiation potential and shortened telomeres. Shortened telomeres imply that the SGSC pool has an extensive replicative history, the reason for which we propose are two-fold.

Firstly, the parenchymal epithelium, e.g. in pSS, non-stem ductal cells and saliva producing acinar cells have been demonstrated to undergo enhanced levels of apoptosis, from sources intrinsic and extrinsic to the cells themselves. Extrinsically, the action of cytokines, cytotoxic T cells and NK cells all promote apoptosis 26. Additionally, a disorganized extracellular matrix in pSS salivary glands may account for acinar cell loss by anoikis 27. Intrinsically, epithelial cells have been recently demonstrated to express defective levels of the anti-inflammatory mediator PPARɣ, resulting in increased activity of the NFκB and IL-6 pathways, but also rendering them more susceptible to cell death 28–33. Similarly, levels of the ubiquitin-editing protein A20, a negative regulator of NFkB was down-regulated in salivary gland epithelial cells from pSS patients compared to healthy subjects 34. Depletion thus of the parenchymal cell pool via intrinsic and extrinsic mechanisms together likely stimulate SGSC proliferation and differentiation into acinar cells, in an attempt to maintain the saliva producing capacity of the SGs.

Secondly, as we have demonstrated here, proinflammatory cytokines exert a direct effect on proliferation of SGSCs. In other model systems, and most extensively in the well characterized intestinal stem cell niche, proinflammatory cytokines have also been reported to exert a proliferative effect, mediated by modulation of the stem cell associated Wnt, Notch and YAP-TAZ pathways, suggesting that cross-talk between stem cells and the elements of the immune system

may underlie many disease manifestations 35–39. Cytokine production in the case of pSS may be derived from neighboring epithelial cells signaling in a paracrine fashion. The production and secretion of pro-inflammatory cytokines by epithelial cells has been demonstrated in long term epithelial culture systems and in situ 31,40–43. Following release of damage- and pathogen-associated molecular patterns (DAMPS/RAMPs), for example molecules such as HMGB1 and viral antigens, pattern recognition receptor (PRRs) on epithelial cells may be activated, culminating in epithelial cell autonomous NFkB pathway activity, cytokine production and paracrine signaling to neighboring SGSCs 32,33. Indeed, the dysregulated NFkB pathway seen in pSS may account for the sustained pro-inflammatory cytokine production by glandular epithelial cells 32,34.

In a healthy scenario, one prevailing stem cell theory dictates that salivary gland stem cells reside in the striated ducts, proliferate and differentiate into intercalated ducts, and then finally into saliva-producing acinar cells. We have shown presence of senescent cells in intercalated ducts of pSS salivary glands. This suggests a blockade in ability of SGSCs to further differentiate into acinar cells, presumably due to having reached their regenerative limit, similar to poor mature organoid differentiation potential we demonstrate in vitro. Clinically, our data suggest that screening patient SGs or saliva for senescence biomarker expression may indicate the extent of SGSC exhaustion. We predict further that clinical interventions aimed at preventing hyposalivation development need to occur before appearance of high levels of senescent markers in SGs or saliva. The present study also suggests, critically, that effective interventions to cure established hyposalivation by targeting the inflammatory process are not likely to involve only immune signal blockade; rather the replenishment of SGSCs stocks in conjunction with resolving the inflammation. Plausible strategies include SGSC manufacture using induced pluripotent stem cell technologies.

In summary, we show for the first time an ageing phenotype as a potential causative agent for the lack of SG repair in the auto immune disease pSS, and link this to possible future clinical strategies.

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Figure legends:

Fig. 1. Salivary gland stem cells from pSS patients show reduced regenerative potential. **A**) Microscopy of primary spheres isolated from HC and pSS biopsies. White arrows denote organoids. **B**) Primary sphere yield quantification per $EpCAM^{hi}$ cell in SGSC isolate from pSS biopsies and HCs. $n = 6$ for HCs, 9 for pSS biopsies. *= $p < 0.05$, student's *t*- test. **C**) Microscopy of organoid cultures from HC and pSS SGSCs. **D**) Organoid forming efficiency of HC and pSS self-renewal cultures. n=27 for HC at all passages. n=12, 16, 9 and 6 for pSS at passages 1-4 respectively. Bar height represents mean, error bars are S.E.M. *=p<0.05,**=p<0.01. Two-way ANOVA and Bonferroni post-hoc testing. **E**) Cumulative population doublings of HC and pSS SGSCs. $n = 26$ for HC passages 1-3 and 24 for passage 4. n=10,5,4 and 2 for pSS at passages 1-4 respectively. Bar height represents mean, error bars are S.E.M. **=p<0.01. Two way ANOVA and Bonferroni post-hoc testing. **F**) Mature organoids formed from a HC SGSC in phase contrast microscopy. **G**) Immunocytochemical staining of acinar cell associated amylase, in a HC derived mature organoid. Inset shows control without anti-amylase antibody to demonstrate staining specificity. **H**) Attempted mature organoid formation from pSS SGSCs.

Fig. 2. Salivary gland stem cells from pSS patients are more likely to be senescent. **A**) Primary sphere yield from HC, incomplete pSS and pSS patients. n=73, 10 and 18 for HC, incomplete pSS and pSS groups respectively. pSS group from Fig. 1b is used for comparison. Line represents median. Yellow points represent SGSCs with unusually high yield. **B**) Volcano plot resulting from RNASeq analysis comparing HC and incomplete pSS

SGSC transcriptomes. Orange box denotes genes whose expression is ≥ Log10 2-fold higher in pSS SGSCs, with a $p<0.01$. **C**) Upregulated cell cycle progression promotion (green), and inhibition (red) genes identified from RNASeq, including the β-galactosidase-like gene (GLB1L2; blue). Dashed line represents mean HC expression. n=6 HCs, 3 pSS. **D**) Raw expression values for GLB1L2. **E**) STELA analysis of SGSC telomere lengths from biopsy-positive pSS patients and HC biopsies show outlying small (<4.5 kb) telomeres in pSS SGSCs samples. **F**) Quantification of telomere lengths in healthy control and pSS SGSCs. Red text denotes percentage of telomeres with a length <4.5kb. **G**) Length analysis of lowest 10% of telomeres in HC and biopsy positive pSS patient SGSCs. n=3 patients per group. Bar height represents mean, error bars are S.E.M. Student's t-test. *= $p\leq0.05$.

Fig. 3. Parotid SGSC organoid cultures proliferate upon exposure to a proinflammatory cocktail and express cell cycle and senescence genes. $p16^+$ senescent cells localize to the intercalated ducts of incomplete pSS and pSS SG tissue. **A**) Phase contrast microscopy of healthy SGSCs at passages 1-4, incubated with (+cytokines) and without (control) the proinflammatory cytokine cocktail. **B**) Quantification of organoid formation efficiency of SGSCs exposed to proinflammatory cytokines, compared to control cells. $n \geq 7$ separate patient isolations at each passage. Error bars represent SEM. * $p<0.05$, *** $p<0.001$, Two Way ANOVA. **C**) Expression of cell cycle associated genes in SGSCs exposed to proinflammatory cytokines. Cells were harvested at the end of passage 1 for qPCR analysis. n=2 separate patient isolations. Error bars represent SEM. **D-G**) Immunohistochemical staining for p16 senescence marker in incomplete pSS (**D,E**), pSS (**F**) and healthy control (HC, **F**) tissue, counterstained with epithelial growth factor receptor (EGFR) to mark all

ductal cells. **G** is high resolution images of red boxed area in **E**. ID = intercalated duct. Ages of tissue donors were 50, 73 and 31 years respectively for HC, incomplete pSS and pSS, indicating that increased $p16^+$ intercalated duct cells was not due to advanced age of donor.