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Association of G protein-coupled receptor 78 with salivary dysfunction in male Sjögren's patients

Tsutomu Tanaka[1] | Maria C. Guimaro[1] | Hiroyuki Nakamura[1] | Paola Perez[2] | Youngmi Ji[1] | Drew G. Michael[1] | Sandra A. Afione[1] | Changyu Zheng[1] | Corinne Goldsmith[1] | William D. Swaim[1] | Anne Marie Lynge Pedersen[3] | John A. Chiorini[1]

[1]Adeno-Associated Virus Biology Section, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, Maryland, USA

[2]Salivary Disorder Unit, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, Maryland, USA

[3]Oral Medicine and Oral Pathology, Department of Odontology, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark

Correspondence
John A. Chiorini, AAV Biology Section, NIDCR/National Institutes of Health, 10 Center Drive, Bethesda, Maryland 20892, USA
Email: jchiorini@dir.nidcr.nih.gov

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Abstract

Objective: Sjögren's disease (SjD) has a strong sex bias, suggesting an association with sex hormones. Male SjD represents a distinct subset of the disease, but the pathogenic mechanisms of male SjD is poorly characterized. The aim of this study is to identify initiating events related to the development of gland hypofunction and autoimmunity in male SjD patients.

Materials and methods: Human minor salivary glands were transcriptomically analyzed with microarrays to detect differentially expressed genes in male SjD patients. Identified genes were tested on their involvement in the disease using conditional transgenic mice and gene-overexpressing cells.

Results: GPR78, an orphan G protein–coupled receptor, was overexpressed in the salivary glands of male SjD patients compared with male healthy controls and female SjD patients. Male GPR78 transgenic mice developed salivary gland hypofunction with increased epithelial apoptosis, which was not seen in control or female transgenic mice. In cell culture, GPR78 overexpression decreased lysosomal integrity, leading to caspase-dependent apoptotic cell death. GPR78-induced cell death in vitro was inhibited by treatment with estradiol.

Conclusion: GPR78 overexpression can induce apoptosis and salivary gland hypofunction in male mice through lysosomal dysfunction and increased caspase-dependent apoptosis in salivary gland epithelium, which may drive disease in humans.

KEYWORDS
apoptosis, GPR78, lysosome, sex hormone, Sjögren's syndrome

1 | INTRODUCTION

Sjögren's disease (SjD) is an autoimmune disease associated with loss of exocrine gland function, primarily of the salivary and lacrimal glands, resulting in symptoms of dryness. Patients with SjD also develop hypergammaglobulinemia and autoantibodies against the antigens Ro/SSA (Ro52 and Ro60) and La/SSB. Systemic symptoms have also been reported and include interstitial lung disease, cutaneous vasculitis, and lymphadenopathy (Odani & Chiorini, 2019). Although the etiology of the disease is not completely clear, disease initiation is supposedly the result of a combination of genetic and environmental factors. This combination of events leads to the

Tsutomu Tanaka, Maria C. Guimaro and Hiroyuki Nakamura contributed equally to this work.

activation of chronic inflammation and tissue damage (e.g., through apoptosis) of the exocrine glands, with fibrosis and loss of physiological function as a result (Odani & Chiorini, 2019).

Sjögren's disease shows a strong sex bias: 90% of patients are female. It has been postulated that this bias is associated with differential immune regulation, X chromosome gene dosage, and changes in sex hormone levels (Qin et al., 2015). In autoimmune diseases with a sex bias, there is often a difference in clinical presentation, with males showing a more significant disease. This can be seen in, for example, systemic lupus erythematosus (Aranow et al., 2002), systemic sclerosis (Hussein et al., 2014), multiple sclerosis (Bergamaschi, 2007), and also in SjD (Ramírez Sepúlveda et al., 2017). In a study of almost 200 cases (93% female), male SjD patients were younger, had higher anti-Ro52 autoantibody levels, and showed a higher incidence of extra glandular manifestations than female patients (Ramírez Sepúlveda et al., 2017). This suggests that the pathogenic mechanisms of SjD may vary between women and men.

Studies to find an association between pathogenesis and molecular mechanisms have often relied on comparative transcriptomic studies between groups. In this study, we investigated the transcriptomic changes associated with the presence of SjD in men utilizing RNA isolated from minor salivary glands (MSGs). In glands from male SjD patients, we found increased levels of GPR78, an orphan G protein–coupled receptor, compared with those from male healthy controls and female SjD patients. Overexpression of GPR78 induced a decrease in lysosomal integrity, which resulted in caspase-dependent apoptosis. This GPR78-associated cell death could be inhibited by estradiol treatment. Our results indicated that GPR78 may be central to cell death and subsequent gland hypofunction in male SjD patients.

2 | MATERIALS AND METHODS

2.1 | Transcriptome analysis

Labial MSGs were surgically excised from SjD patients and age-matched healthy volunteers (HVs) for transcriptome analysis, in accordance with the Declaration of Helsinki principles. Prior to the study, research participants provided informed consent to the protocol, which was approved by the NIH Single Institutional Review (15-D-0051, NCT02327884). Messenger RNA was isolated from each biopsy using the QIAGEN RNeasy Kit (QIAGEN, USA) and DNase-treated using an on-column DNase I digestion to remove contaminating DNA. RNA quality of each RNA extraction was evaluated on an Agilent Bioanalyzer using the RNA 6000 Nano Chip (Agilent, USA). Only samples with RIN > 7 were accepted into the study.

Microarray analysis was performed per the manufacturer's standard protocol (Agilent). Following feature extraction, data were normalized by performing a 75th percentile shift, and probes h that had been subjected to igh levels of noise (<20th percentile expression) were removed from downstream analyses (File S1).

Each gene expression level was compared between SjD patients and HVs using Student's *t*-test to find significantly upregulated genes (defined as unadjusted *p*-value <0.01, fold change >2) in patients' MSGs.

2.2 | Transgenic mice

The transgenic vector pCLE-GPR78 was microinjected into the pronucleus of fertilized FVB/N mouse oocytes. EGFP-expressing transgenic founder mice (pCLE-GPR78) were mated with wild-type FVB/N mice (Envigo, USA) to create a founder mouse generation. GPR78 transgenic mice were initially screened by visualization of GFP expression in exposed tissue using a flashlight source and filters. All GPR78 transgenic mice were genotyped by PCR (forward primer: 5′- AAT ACG ATC GGA ATT CGA CAC CAT AGG GCG GCC GGG AAT TCG -3′; reverse primer: 5′- GCC TGC ACC TGA GGA GTG AAT TGC TAG CGG CCG GCC GTT TAA ACC TTA TCG T -3′) from tail snip DNA using the JumpStart REDTaq ReadyMix Reaction Mix (#P0982, Sigma-Aldrich). Adenoviral vectors expressing CRE recombinase or luciferase were delivered into both submandibular glands of each 9-week-old mouse by retrograde cannulation using 1×10^8 particles/gland. All procedures involving live animals were approved based on institutional guidelines and standard operating procedures following the NIH Guide for the Care and Use of Laboratory Animals (approval number: 18-863). A detailed explanation of transgenic vector construction, CRE-LoxP system and histological assessment is listed in the online Supplemental Materials and Methods.

2.3 | Cell assays

Details on cell culture, transient transfection, cell cycle analysis, western blotting, quantitative real-time reverse transcription PCR, apoptosis assay, cytoplasmic cathepsin B activity measurement, galectin-3 puncta assay can be found in the online File S1.

2.4 | Statistical analysis

Student's *t*-test, Tukey's or Dunnett's multiple comparison test were used for analysis of in vivo and in vitro studies. These analyses were carried out with GraphPad Prism 8 (GraphPad Software, USA). A *p*-value <0.05 was regarded as statistically significant. All statistical tests were two-sided.

3 | RESULTS

3.1 | GPR78 is overexpressed in minor salivary glands of male SjD patients

To identify gene expression changes that may be associated with the presence of SjD in males, we analyzed 24 human MSGs (2 male SjD, 7 female SjD, 5 male HVs, and 10 female HVs) using high-density microarrays. The sex of each sample was confirmed by the expression

of *RPS4Y1*, *DDX3Y*, and *RPS4Y2*. Both the male patients showed a significant decrease in unstimulated salivary flow rate (<0.1 mL/min) and had lymphocytic foci in their salivary glands. One male patient had serum autoantibodies against Ro/SSA and La/SSB (Table S1).

Genes that were differentially expressed between male SjD patients and HVs were identified based on statistical significance ($p < 0.01$ following 75th percentile shift normalization). Among the 161 genes differentially expressed at greater than 2-fold levels between the male SjD patients and HVs, *GPR78* was the most significantly upregulated gene (Figure 1a). Normalized expression of the signal detected on additional microarrays from female patients and HVs suggested that GPR78 expression is elevated in only males with SjD (Figure 1b). Confirmatory confocal immunofluorescent imaging demonstrated increased GPR78 expression in a male SjD patient compared with an HV (Figure 1c).

3.2 | GPR78 overexpression induces apoptosis in vitro

GPR78 is an orphan receptor with an unknown ligand and a significant level of constitutive cAMP activity. Expression of GPR78 has been reported in the central nervous system and is associated with susceptibility to bipolar affective disorder and schizophrenia (Underwood et al., 2006). Nonetheless, little is known about its role in epithelia.

Previously, GPR78 expression was reported to significantly reduce cell growth compared with controls in 293T cells (Guimaro et al., 2020). We, therefore, performed cell cycle analysis using propidium iodide and flow cytometry, which showed a small but statistically significant increase in the length of the S phase of GPR78-overexpressing 293T cells compared with control 293T cells (Figures S1a,b). However, this change was likely too small to account for the reduction in cell growth previously reported (Guimaro et al., 2020).

Next, we examined GPR78-overexpressing 293T cells for apoptotic cell death. GPR78-overexpressing 293T cell culture showed a significant dose-dependent increase in the number of Annexin V^+ cells compared with the control 293T cell culture as measured by APC Annexin V/7-AAD staining and flow cytometry (Figures S1c), suggesting that GPR78 overexpression induces apoptosis.

3.3 | GPR78 transgenic mice develop salivary gland hypofunction

Excessive and inappropriate cell death has been proposed to be an early event in the pathogenesis of SjD. As was recently shown,

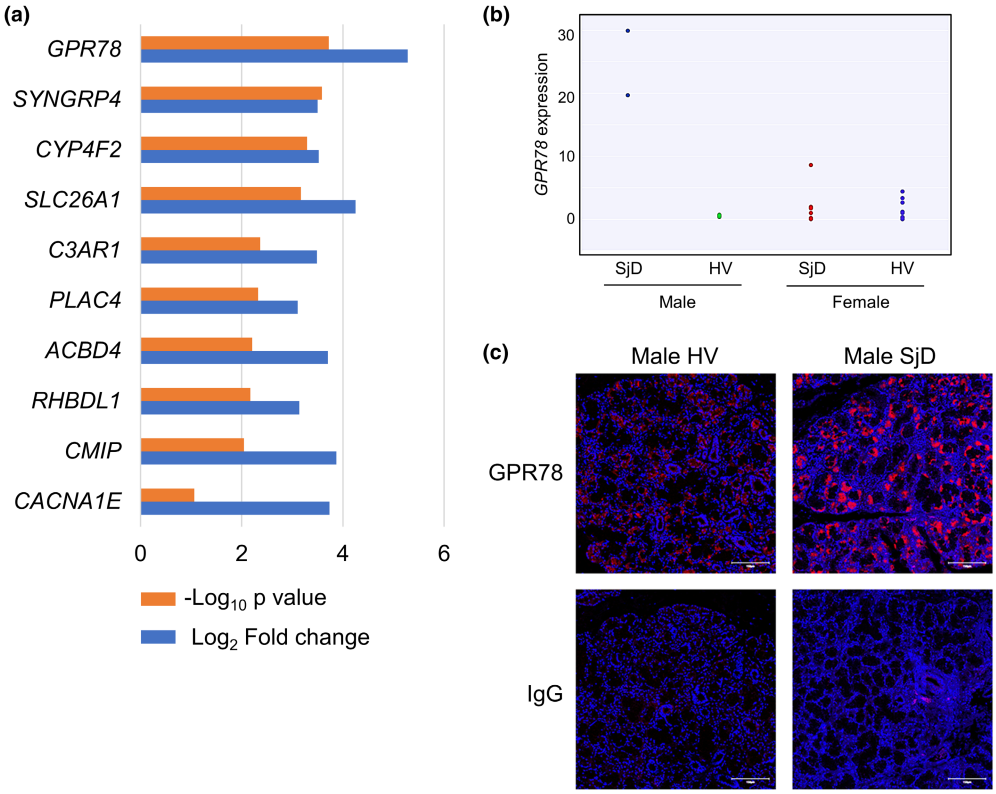

FIGURE 1 GPR78 is overexpressed in minor salivary glands of male Sjögren's disease patients. (a) Top 10 upregulated genes in minor salivary glands of male patients with Sjögren's disease (SjD) ($n = 2$) compared those with male healthy volunteers (HVs) ($n = 5$). (b) Normalized *GPR78* expression levels in minor salivary glands of male SjD patients ($n = 2$), female SjD patients ($n = 7$), male HVs ($n = 5$), and female HVs ($n = 10$). (c) Representative immunofluorescent images of minor salivary glands from a male SjD patient or HV (scale bar: 100 μm).

enhanced apoptosis in gland epithelia can trigger SjD-like autoimmunity in mice (Nakamura et al., 2021; Okuma et al., 2013). To investigate the pathophysiological role of GPR78 expression and assess if there is a causal or secondary relationship with SjD, we established a mouse model with floxed GPR78 (pCLE-GPR78 vector), in which local GPR78 expression in the submandibular gland could be induced by retroductal cannulation with an adenovirus vector encoding CRE recombinase (Ad-CRE). Transfection of 293T cells with pCLE-GPR78 with or without CRE recombinase showed expression of GPR78 only in the presence of CRE recombinase (Figure S2a), confirming the regulated expression of the construct.

A transgenic mouse line was established by microinjecting pCLE-GPR78 vector into the pronucleus of fertilized FVB/N mouse oocytes. In the "off state", pCLE-GPR78 encodes *EGFP*, and GFP expression was therefore used to screen newborn mice prior to confirmation by genotyping. Following cannulation and retrograde infusion of Ad-CRE, GPR78 expression was detected by RNA in situ hybridization in the submandibular glands of transgenic mice (Figure S2b). Induction of GPR78 expression by Ad-CRE adenoviral transduction of the salivary glands did not appear to have an overt effect on food intake by the mice, as there was no significant difference in body weight between Ad-CRE–treated mice and control mice (Figure S3a).

The effect of GPR78 expression on salivary gland cell apoptosis was tested 4 weeks after cannulation with Ad-CRE by TUNEL assay and light microscopy. Apoptotic TUNEL$^+$ cells were abundant in the salivary glands from male GPR78 transgenic mice (Figure 2a). Overall, the number of apoptotic cells was significantly increased in male GPR78 transgenic mice compared with male control mice (112 vs. 7 per mm^2, $p<0.01$, Figure 2b). Despite the presence of GPR78 mRNA transcripts, as shown by RNA in situ hybridization, TUNEL$^+$ cells were rare in female mice, and the number of apoptotic cells was

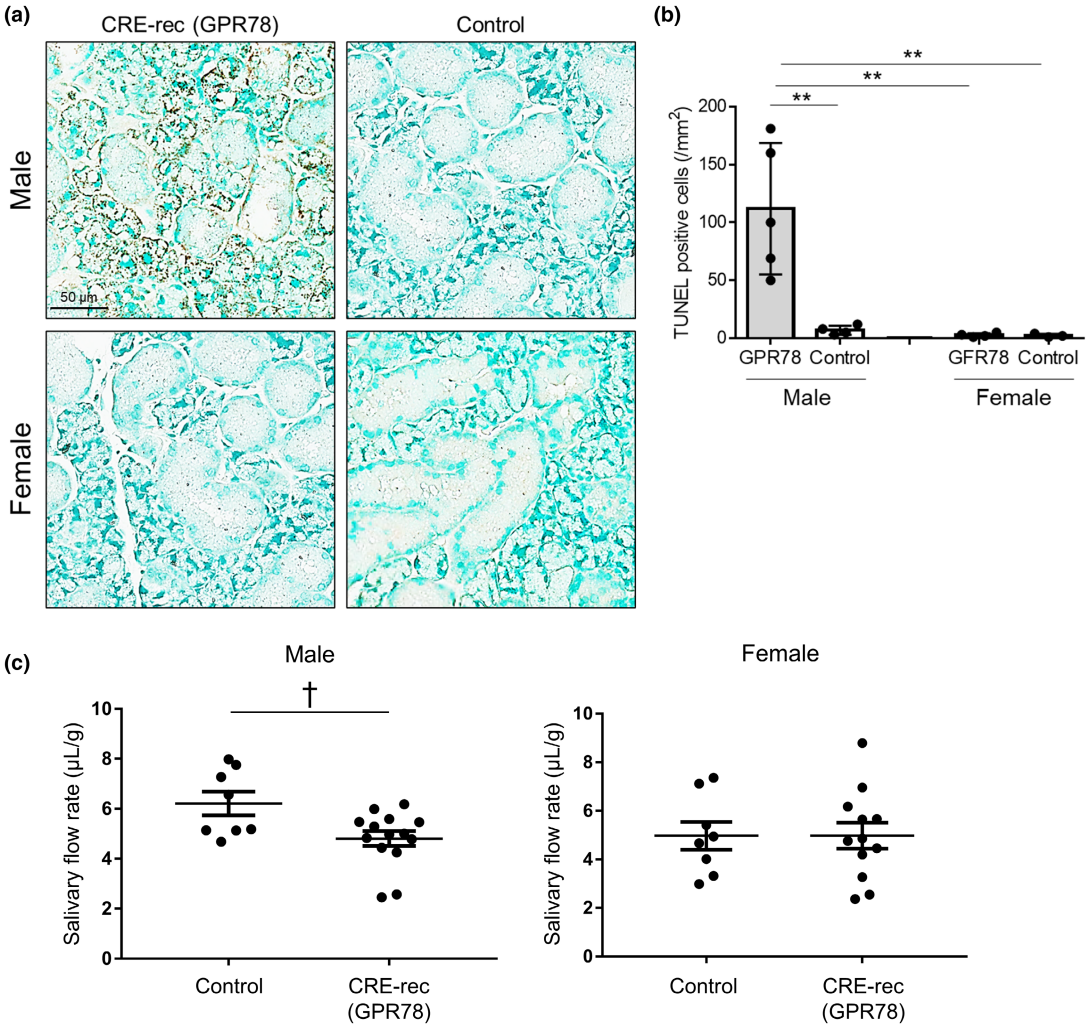

FIGURE 2 Male GPR78 transgenic mice develop apoptotic cell death and have salivary gland hypofunction. Conditional GPR78 expression was induced in murine submandibular glands using CRE-LoxP system. (a) Representative images of terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining of submandibular glands from male and female GPR78 transgenic mice, compared with control mice. Brown staining indicates apoptotic cells. (b) Number of TUNEL-positive (apoptotic) cells was quantified. (c) Pilocarpine-stimulated salivary flow rate per g body weight in 20 min. Values are shown as mean ± SD. $^{**}p<0.01$ based on *t*-test with Tukey's multiple comparison test. $^{\dagger}p<0.05$ based on unpaired Student's *t*-test.

similar to that in control female mice (2.8 vs. 2.3 per mm^2, Figure 2b). These results suggested that GPR78 can induce apoptosis in vivo and that male mice are more sensitive to the effects of GPR78 than female mice.

The effect of GPR78 expression on salivary secretion was also tested in GPR78 transgenic mice 4 weeks after cannulation by stimulating salivary flow with pilocarpine and collecting the saliva for 20 min. Salivary gland function was inhibited in a sex-dependent manner: the salivary flow rate was significantly decreased in male GPR78 transgenic mice compared with male control mice, but not in female GPR78 transgenic mice (Figure 2C). Little difference in focus score (Figure S3b) was observed. These findings suggested that GPR78 can directly affect salivary gland function by induction of epithelial apoptosis in vivo.

3.4 | GPR78-induced apoptosis depends on increased lysosomal membrane permeabilization

As shown above, GPR78 expression induced an increase in apoptotic cell death. However, the mechanism behind this is still unclear. To examine this in detail, we used HSG cells, human epithelial cells (Figure 3a).

GPR78 contributes to the production of cAMP, an intracellular second messenger that is often involved in both pro- and anti-apoptotic signaling (Insel et al., 2012; Jones et al., 2007). Treatment of HSG cells with forskolin, which is a well-known drug used to raise cAMP levels, led to a significant increase in cell death (Figure S4a).

Caspases are well-known initiators of cell death by apoptosis (Clarke & Tyler, 2009; Li et al., 2018). Treatment with the pan-caspase inhibitor zVAD-fmk (Z-VAD) resulted in a significant decrease in cell death in GPR78-overexpressing HSG cells (Figure 3b,c), indicating that GPR78-induced cell death is caused by caspase-dependent induction of apoptosis.

Reactive oxide species (ROS) are synthesized aberrantly in SjD (Saito et al., 2014), and they act as a trigger of apoptosis via caspase activation (Fang et al., 2007). To study whether GPR78 induces this increased ROS production, GPR78-overexpressing HSG cells were probed with CM-H_2DCFDA, a cell-permeant indicator for ROS. No significant difference in ROS level was seen between GPR78-overexpressing HSG cells and control HSG cells (Figure S4b).

Endoplasmic reticulum (ER) stress is increased in minor salivary glands from SjD patients (Barrera et al., 2016) and is also known to be linked to apoptosis via caspase activation (Egger et al., 2007; Martín-Pérez et al., 2014). To test the effect of GPR78 overexpression on ER stress, mRNA expression level of two ER stress markers, *ATF4* and *CHOP*, were analyzed by using qRT-PCR. Although the *ATF4* mRNA level was significantly decreased in GPR78-overexpressing HSG cells compared with control HSG cells (Figure S4c), the *CHOP* mRNA level was significantly increased by GPR78 overexpression (Figure S4d).

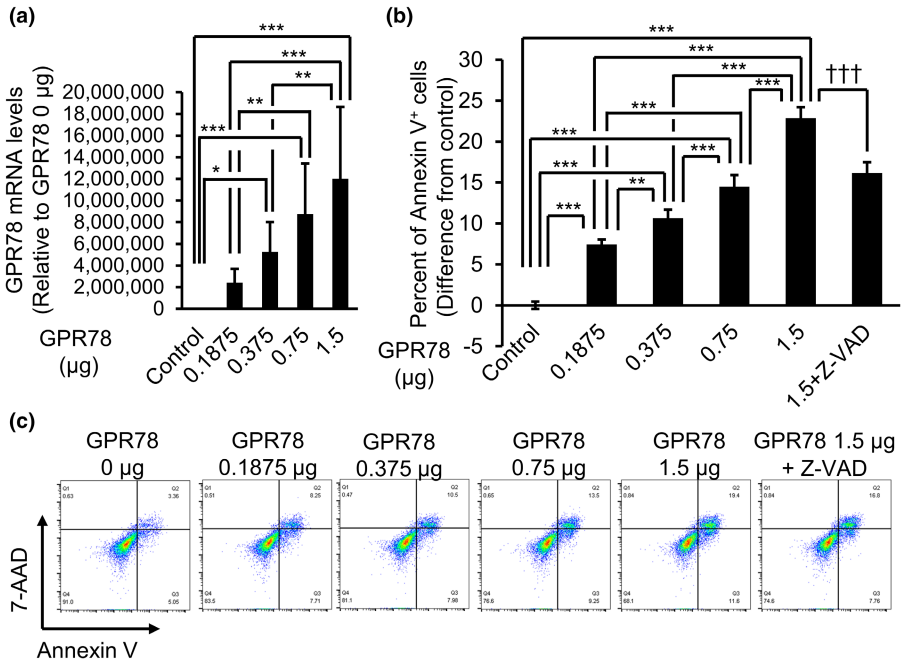

FIGURE 3 GPR78 overexpression induces cell death via caspase-dependent pathway. HSG cells were transfected with GFP control or GPR78-GFP plasmid. (a) *GPR78* mRNA expression levels in control and GPR78-overexpressing HSG cells measured by qRT-PCR. Values are presented as relative change compared with controls ($n = 3$). (b,c) HSG cells were treated with or without 20 μM zVAD-fmk (Z-VAD) 48 h post-transfection, and number of apoptotic cells in GFP-positive population was measured 24 h after incubation by flow cytometry using APC Annexin V/7-AAD. Difference in number of Annexin V+ cells in GPR78-overexpressing cell culture treated with or without Z-VAD compared with control cell culture is shown ($n = 4$). Values are shown as mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ based on Tukey's multiple comparison test. $^{\dagger\dagger\dagger}p < 0.001$ based on unpaired Student's *t*-test.

Lysosomal membrane permeabilization (LMP) induces caspase activation, which results in apoptosis (Johansson et al., 2010; Noguchi et al., 2020). Lysosomal-associated membrane protein 1 (LAMP1) and 2 (LAMP2), as well as 70-kDa heat shock protein (HSP70), are required for lysosomal membrane integrity, which inhibits LMP (Fehrenbacher et al., 2008; Kirkegaard et al., 2010; Noguchi et al., 2020). Western blot analysis of HSG cells indicated GPR78 overexpression leads to decreases in LAMP1 and LAMP2 protein levels compared with control HSG cells, while GPR78-overexpressing HSG cells exhibited no difference in HSP70 levels compared with control HSG cells (Figure 4a). Both *LAMP1* and *LAMP2* mRNA expression levels showed a significant decrease of approximately 30% in GPR78-overexpressing HSG cells compared with control HSG cells (Figure 4b).

To further study the effect of GPR78 on lysosomal membrane stability, the degree of lysosomal membrane damage was compared using a galectin-3 puncta formation assay. GPR78-overexpressing HSG cells showed a significant increase in the percentage of galectin-3 puncta^{+} cells compared with control HSG cells (Figure 4c). Since lysosomal membrane instability often results in LMP, the status of lysosomes was monitored using LysoTracker. GPR78-overexpressing HSG cells exhibited a significant reduction in

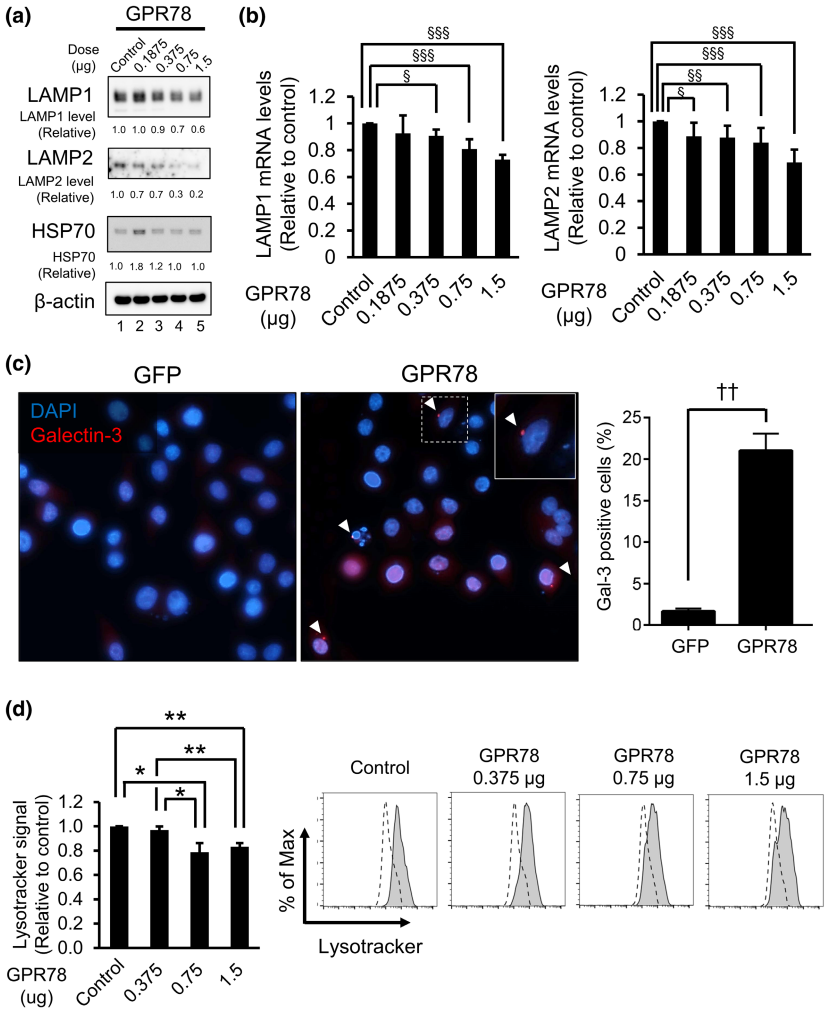

FIGURE 4 GPR78 Overexpression induces apoptotic cell death by lysosomal membrane permeabilization. HSG cells were transfected with 1.5 μg of GFP control plasmid or mixture GPR78-GFP plasmid equaling 1.5 μg of DNA. (a) Western blot analysis of LAMP1, LAMP2, and HSP70 protein expression in GFP-expressing HSG cells (control) and GPR78-overexpressing HSG cells. Protein levels were normalized to β-Actin level. (b) LAMP1 and LAMP2 mRNA expression levels in GFP-expressing HSG cells (control) and GPR78-overexpressing HSG cells were measured by qRT-PCR. Relative levels compared with control are shown ($n = 3$). (c) Representative images of galectin-3 puncta staining of GFP-expressing HSG cells (control, left panel) and GPR78-overexpressing HSG cells (right panel) using immunofluorescent analysis. Percentage of galectin-3 puncta–positive cells per 100 GFP- or GPR78-overexpressing cells is shown ($n = 4$). (d) GFP-expressing HSG cells (control) and GPR78-overexpressing HSG cells were analyzed 72 h post-transfection using flow cytometry with LysoTracker Red DND-99 (white area with dashed line: Background signal; gray area with solid line: Stained cells). Data are presented as relative change in mean fluorescent intensity compared with control ($n = 3$). Values are shown as mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$ based on Tukey's multiple comparison test. $^{\S}p < 0.05$, $^{\S\S}p < 0.01$, $^{\S\S\S}p < 0.001$ based on Dunnett's multiple comparison test. $^{\dagger\dagger}p < 0.01$ based on unpaired Student's *t*-test.

LysoTracker staining, which reflects an increase in the lysosomal pH and is therefore indicative of LMP (Figure 4d). Taken together, these results suggest that GPR78 is able to induce a decrease in lysosomal membrane integrity.

Cytoplasmic cathepsin B activity was elevated in GPR78-overexpressing HSG cells compared with control HSG cells (Figure 5a), suggesting that GPR78 induces LMP, which in turn leads to relocalization of cathepsin B to the cytoplasm. Treatment with E64D, a cathepsin B inhibitor, induced a significant decrease in apoptosis in GPR78-overexpressing HSG cells (Figure 5b). In addition to cathepsin B, cathepsin D—also a lysosomal enzyme—is associated with apoptosis by LMP (Boya & Kroemer, 2008; Song et al., 2017). Treatment with pepstatin A, a cathepsin D inhibitor, showed a significant decrease in apoptosis in GPR78-overexpressing HSG cells (Figure 5c). Taken together, the results suggested that GPR78 induces an increase in apoptosis via LMP by relocalization of lysosomal enzymes.

3.5 | Estradiol treatment prevents apoptosis induced by GPR78

As shown above, GPR78 overexpression led to a significant decrease in salivary flow rate and an increase in the number of apoptotic cells in submandibular glands of male GPR78 transgenic mice, but not in those of female mice. To test our hypothesis that female hormones prevent GPR78-induced apoptosis, we used MCF7 cells, which is a breast cancer cell line that expresses the estradiol receptor. GPR78 overexpression in MCF7 cells resulted in caspase-dependent apoptosis (Figure S5a–c), similar to what we observed in HSG cells (Figure 3a–c). GPR78-overexpressing MCF7 cells also showed decreases in LAMP1 and LAMP2 protein levels, which were inhibited by estradiol treatment, while no difference in HSP70 protein level was found between GPR78-overexpressing MCF7 cells and control MCF7 cells (Figure 6a). Moreover, treatment with estradiol led to a significant dose-dependent decrease in apoptosis

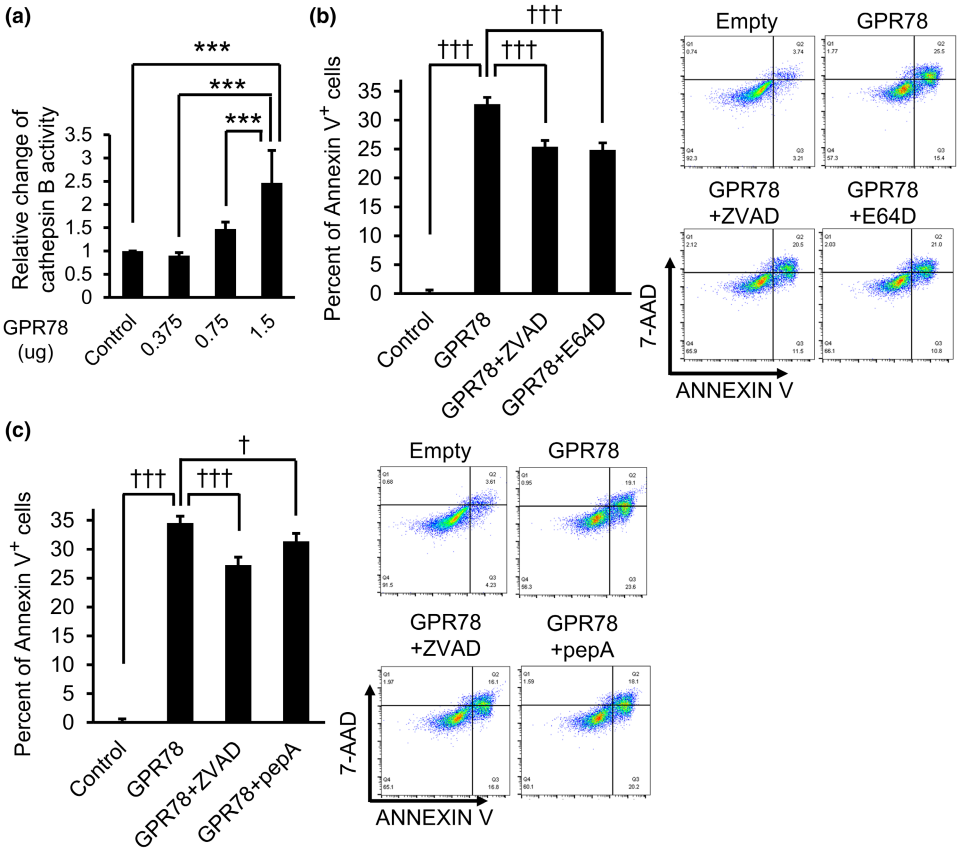

FIGURE 5 GPR78-induced apoptosis is dependent on cathepsin B and D activity. HSG cells were transfected with GFP control or GPR78-GFP plasmid. (a) Cathepsin B activity in cytoplasm of GFP-expressing HSG cells (control) and GPR78-overexpressing HSG cells was measured. Values were normalized to loaded protein concentration, and relative change in activity compared with control cells is shown ($n = 3$). (b) GFP-expressing HSG cells (control) and GPR78-overexpressing HSG cells were incubated with or without 20 μM Z-VAD or 2.5 μM E64D for 14 h. Number of apoptotic cells in GFP-positive population was determined by flow cytometry using APC-Annexin V/7-AAD. Difference in number of Annexin V+ cells compared with control is shown ($n = 3$). (c) GFP-expressing HSG cells (control) and GPR78-overexpressing HSG cells were incubated with or without 20 μM Z-VAD or 5 μM pepstatin A (pepA) for 14 h. Number of apoptotic cells in GFP-positive population was determined by flow cytometry using APC-Annexin V/7-AAD. Difference in number of Annexin V+ cells compared with control is shown ($n = 3$). Values are shown as mean ± SD. $^{***}p < 0.001$ based on Tukey's multiple comparison test test. $^{\dagger}p < 0.05$, $^{\dagger\dagger\dagger}p < 0.001$ based on unpaired Student's *t*-test.

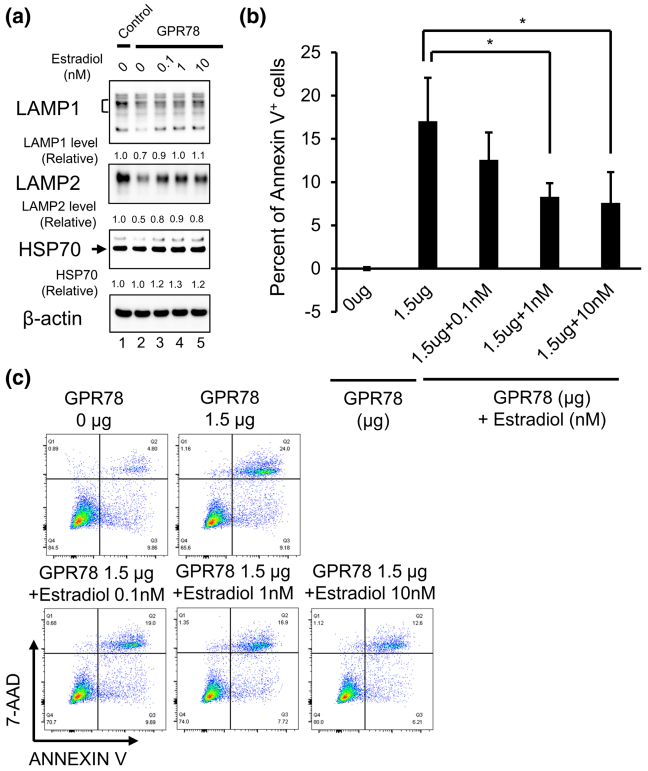

FIGURE 6 GPR78-Induced apoptosis and decreased lysosomal membrane protein levels are inhibited by estradiol. MCF7 cells were transfected with GFP control or GPR78-GFP plasmid and were treated with or without estradiol 48 h post-transfection. (a) Western blot analysis of LAMP1, LAMP2, and HSP70 protein expression in MCF7 cells. Protein levels were normalized to β-Actin level. (b,c) Number of apoptotic cells in GFP-positive population was determined 24 h after incubation with estradiol by flow cytometry with APC Annexin V/7-AAD. Difference in number of Annexin V+ cells compared with control is shown ($n = 4$). Values are shown as mean ± SD. $^*p < 0.05$ based on Dunnett's multiple comparison test.

in GPR78-overexpressing MCF7 cells (Figure 6b,c). This result suggested that estradiol in the female mice might inhibit GPR78-induced apoptosis by LMP resulting in preservation of salivary flow rate.

4 | DISCUSSION

Sjögren's disease patients have a heterogeneous clinical presentation, and a variety of molecular subsets of patients are likely responsible for the lack of universal predictive biomarkers and effective treatments. However, one common feature is the strong sex bias in the presence of this disease, which indicates that male SjD represents a very unique subset of patients. Through our analysis of the transcriptome of these male patients, we found an upregulation of a little-studied protein, GPR78. Then, we showed that GPR78 overexpression can induce LMP, apoptosis and salivary gland hypofunction. Despite the unique expression of GPR78 in male SjD patients compared with female SjD patients, the development of disease parallels much of what we know about SjD in women: The disease presumably begins with apoptosis. We have recently described that apoptosis is induced by lysosomal dysfunction and leakage of cathepsins into the cytoplasm following LMP (Tanaka et al., 2020, 2021). This is similar to our observations of induction of LMP following overexpression of GPR78. GPR78-induced apoptosis could be blocked by estradiol treatment supporting the unique expression GPR78 exclusively in male SjD patients.

Although male and female minor salivary glands are functionally similar, significant differences exist between their transcriptomes. Previous work has described almost 800 genes that were differentially expressed between male and female parotid glands (Srivastava et al., 2008). Many of these differences are probably a direct result of sex hormones on their receptors. Other researchers have proposed that some estrogen-responsive genes are downregulated in healthy females compared with healthy males (Bale et al., 2001; Chang et al., 1999; Larcher et al., 1995; Takahashi et al., 1994; Treister et al., 2005). In contrast, many genes upregulated in females compared with males have a stimulatory effect on the androgen receptor (Takahashi et al., 2001; Yang et al., 2007). Taken together, these data indicate a difference in estrogen receptor signaling between female and male salivary glands by an inhibitory relationship between the estrogen and androgen receptors. This is particularly interesting because most women develop SjD in their perimenopausal years. One can hypothesize that a loss of the delicate sex hormone balance can lead to an increased risk of SjD. Significantly higher levels of prolactin have been reported in SjD patients, as well as significantly higher prolactin/progesterone and estrogen/progesterone ratios (Taiym et al., 2004). This theory is consistent with observations that the conversion of dehydroepiandrosterone to androgen, which is the main source of androgens in the salivary glands in postmenopausal women, is impaired in women with SjD compared with age-matched healthy women (Laine et al., 2007; Porola et al., 2007, 2008). While it has been proposed that androgens protect against SjD, in this study, we have demonstrated that estradiol treatment protects MCF7 cells against GPR78-induced apoptosis via LMP. Estradiol prevents apoptosis following hypoxic stress in cardiomyocytes (Kim et al., 2006) and plays a role in balancing lysosomal activities through many mechanisms, ranging from transcriptional modulation to miRNA expression and histone modification (Xiang et al., 2019).

An intriguing aspect of our study is the lack of lymphocyte infiltration despite the induction of salivary gland hypofunction in animals receiving local Ad-CRE. Although we monitored the mice for 8 weeks after viral vector delivery and observed little change in immune activation, in many other SjD mouse models, mice are at least 40 weeks of age before they develop markers of systemic immune activation (Groom et al., 2002). Recently, we have shown that LMP-induced autoimmunity required 16 weeks following induction of the causative gene expression in mice (Nakamura et al., 2021). It is possible that following apoptosis, more time is required for the development of autoimmunity through a GPR78-dependent pathway. Additional research is required to determine if GPR78 transgenic mice represent a model for salivary gland hypofunction or SjD with associated autoimmunity.

A limitation of this study is that mice lack a GPR78 orthologue. Although several mouse models of SjD are reported, many involve immunomodulation rather than manipulation of the transcriptome of the murine salivary glands. To limit the immunologic effect of

expressing a foreign human gene in a mouse, we created a transgenic line. However, it is still possible that expression of GPR78, an orphan receptor with constitutive cAMP activity, might interact differently with the murine versus human regulome and affect murine epithelial cell survival more than its gene-specific function in our GPR78 transgenic mouse model. Although male SjD is an extremely rare condition, we were able to examine two unrelated samples which both showed elevated expression of *GPR78* by microarrays, and one was confirmed by immunofluorescence. Future studies in additional subjects are necessary to generalize the findings in male SjD.

In summary, our study on the transcriptome of male SjD patients suggests that while the clinical presentation and molecular mechanisms of SjD are similar for male and female patients, the triggers for disease initiation could be different. Furthermore, the present results demonstrate a critical role for salivary epithelial lysosomes and LMP in the development of SjD.

AUTHOR CONTRIBUTIONS

Tsutomu Tanaka: Conceptualization; data curation; formal analysis; investigation; methodology; visualization; writing – original draft. **Maria Cambraia Guimaro:** Data curation; formal analysis; investigation; methodology; visualization; writing – original draft. **Hiroyuki Nakamura:** Data curation; formal analysis; investigation; visualization; writing – original draft. **Paola Perez:** Data curation; formal analysis; investigation; methodology; visualization; writing – review and editing. **Youngmi Ji:** Methodology; project administration; supervision; writing – review and editing. **Drew Michael:** Data curation; formal analysis; investigation; methodology; writing – review and editing. **Sandra Wainer:** Investigation; methodology; resources; writing – review and editing. **Changyu Zheng:** Data curation; investigation; methodology; supervision; writing – review and editing. **Corinne Goldsmith:** Data curation; investigation; methodology; writing – review and editing. **William Swaim:** Methodology; resources; software; supervision; visualization; writing – review and editing. **Anne Marie Lynge Pedersen:** Conceptualization; project administration; resources; writing – review and editing. **John (Jay) Chiorini:** Conceptualization; project administration; resources; supervision; writing – original draft.

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CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

DATA AVAILABILITY STATEMENT

All relevant data are included in the article and are also available in a public repository.

ORCID

Maria C. Guimaro https://orcid.org/0000-0002-2633-9710
Hiroyuki Nakamura https://orcid.org/0000-0003-2149-7573
Anne Marie Lynge Pedersen https://orcid.org/0000-0002-6424-5803

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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