Supporting Information

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SI Materials and Methods

Cell Culture, BMP6 Treatment, and Transfection. The HSG cell line was cultured with 1× minimum essential medium (GIBCO) with 10% (wt/vol) FBS (Invitrogen) containing 1% antibiotics at 37 °C in 5% (vol/vol) CO_2. Recombinant human BMP6 was purchased from R&D Systems. The stock solution was diluted in 1 mM Tris·HCl buffer containing 0.1% BSA or human serum albumin, and stored at –20 °C. Cells were treated for 72 h with the indicated concentration of BMP6 throughout the study.

Animals, AAV Vector Administration, and Serum/Saliva/Tear Collection. Six- to eight-week old female C57BL/6 mice were obtained from The Jackson Laboratory. Animals were housed in a pathogen-free facility. All mice were housed and cared for in accordance with institutional guidelines. Thirty-week-old C57BL/6.NOD-*Aec1*/*Aec2* mice were housed at the University of Florida under specific pathogen free (SPF) conditions as previously reported (22). The construction and delivery of the AAV vectors used in this study have been described previously (10). Vectors were delivered into the submandibular glands by retrograde cannulation, and saliva, tear, and serum were collected as previously described (22). BMP6 and AQP1 expression was confirmed by confocal imaging at the time of tissue collection.

Microarray Preparation, Statistical Analysis, and RT-PCR. Gene expression analysis involved the use of 4 × 44,000 microarrays (Agilent) containing 41,000 human oligo probes. Microarrays were hybridized according to the manufacturer's recommendations for one-color microarray-based gene expression analysis. Quality control criteria were established based on the results of previously published experiments (10). Total RNA was extracted with an RNeasy Mini Kit according to the instructions of the manufacturer (Qiagen) and processed as previously described (10). Data analysis was completed using GeneSpring (GX 11; Agilent Technologies), including preprocessing of raw data, data normalization, sample quality control, *t* test plus Benjamin–Hochberg correction, clustering, annotation, and accession to biological context. Microarrays and quantitative PCR reactions were completed as described previously (10). Microarray data were normalized using a percentile shift to 75 and a threshold cutoff of 1.0. Microarray data are deposited with the Gene Expression Omnibus (GSE80225).

Measurement of Cell Volume and Recovery of RVD by AQP5 Transfection. HSG cells were loaded with the fluoroprobe calcein (Molecular Probes, Inc.) and excited at 490 nm. Emitted fluorescence was measured at 510 nm. In situ calibration of the dye was performed. The relationship between dye fluorescence and the volume change was linear over the volume range studied (10). The cell volume was estimated using an Olympus 51× microscope interfaced with Universal Imaging MetaMorph software. Data are presented as mean ± SEM. Fluorescence measurements were made using a Till Photonics-Polychrome IV spectrofluorimeter attached to an Olympus 51× microscope and a Metafluor imaging system (Universal Imaging Corp.). RVD was induced by addition of hypotonic solution (HTS) of 150 mOsm, and relative cell volume was measured before and after HTS stimulation. For complementation experiments, HSG cells were grow to 70–90% confluence, and then transfected by using Lipofectamine 2000 according to the manufacturer's instruction (Invitrogen). Briefly, six-well plates containing HSG cells were changed with new growth medium without antibiotics before transfection. Treatment with BMP6 was performed as described above. A total of 3.0 μg of DNA of either AAV2-AQP1 or AAV2-AQP5, as indicated in Fig. 3, was combined with a reporter plasmid encoding dsred (1 μg) as a transfection control and pUC19 as a carrier plasmid and mixed with 50 μL of Opti-MEM Reduced Serum (Invitrogen) and 50 μL of diluted reagent of Lipofectamine 2000 (10 μL per well). After incubation for 20 min at room temperature, the mixture was added to the HSG cells, and the media were changed after 4–6 h. The transfected HSG cells were incubated at 37 °C in a CO_2 incubator for 18–48 h before testing for transgene expression.

Confocal Immunofluorescences. HSG cells were treated with 6 ng/mL BMP6 for 3 d. The HSG cells were then fixed at 37 °C in 4% (vol/vol) formalin for 30 min and immediately washed at 37 °C with PBS buffer. Immunofluorescent detection was done using labeled phalloidin (1:1,000 dilution) and anti-AQP5 (1:1,000 dilution; Alamone Laboratories) and a mouse monoclonal anti-BMP6 (Abcam). Quantification of AQP5 expression in HSG cells was determined by imaging of 293T cells transfected with increasing amounts of AQP5 encoding plasmid as a standard curve. Images were collected with an Olympus FV1200 confocal system using a 100× 1.4-N.A. objective. Patients with high BMP6 are defined as those patients with a mean level of expression 2 SDs above the mean level of expression seen in healthy individuals (10). Phalloidin was conjugated with TRITC. AQP1 in mouse samples and AQP5 in human samples were detected as follows. Paraffin sections were cut at 5 μm and adhered to charged slides. After drying, sections were deparaffinized and rehydrated to water. Antigen unmasking was performed in a microwave oven using 1 mM EDTA (pH 8) with 0.05% Tween 20 for 10 min. After cooling, sections were blocked with 5% (vol/vol) normal donkey serum in 0.5% BSA in PBS for 30 min at room temperature. The blocking solution was removed and replaced with the antibody solution at 0.5 μg/mL anti-AQP5 polyclonal antibody (Santa Cruz Biotechnology) and 10 μg/mL anti-AQP1 rabbit monoclonal antibody (Abcam) in 0.5% BSA in PBS and incubated overnight in a humidified chamber at 4 °C. Negative controls substituted normal nonimmune rabbit IgG at 10 μg/mL for the primary antibody. On the following morning, slides were washed five times for 5 min each time with PBS at room temperature on a stir plate with gentle stirring. Sections were then incubated with secondary antibody (donkey anti-rabbit conjugated with Alexa-488) diluted 1:200 in 0.5% BSA in PBS in a humidified chamber for 1 h at room temperature. Sections were washed as above and coverslipped with Fluoromount G with DAPI and a no. 1.5 glass coverslip. Images were captured with an Olympus Fluoview 1000 confocal system using a 40× oil objective. Patient slides were imaged as previously described (10). Briefly, slides were blocked with 10% (wt/vol) donkey serum in 0.5% BSA, followed by incubation overnight at 4 °C with mouse monoclonal anti–BMP6 primary antibody (Abcam) in 0.5% BSA in PBS and then incubated with Alexa Fluor 488 goat anti-mouse IgG secondary antibody (Invitrogen) for 1 h, followed by counterstaining with DAPI mounting medium. Slides with total fluorescent values 2 SDs above the mean level in the healthy group were considered to have high expression of BMP6.

Flow Cytometry Analysis of Salivary Glands and Serum Cytokine and Chemokine Detection. Single-cell suspensions of salivary gland cells were prepared as previously described (22). The resulting leukocyte suspensions were resuspended in RPMI medium 1640 containing 10% (vol/vol) FBS, 2 mM L-glutamine, and 0.05 mM β-mercaptoethanol to a density of 2×10^6 cells per milliliter. One million cells were pipetted to individual wells of a 24-well microtiter plate precoated with anti-CD3 (10 μg/mL) and anti-CD28 (2 μg/mL; BD Biosciences). Cells were incubated for 5 h with leukocyte activation

mixture containing GolgiPlug (2 μL/mL; BD Biosciences). Collected cells were fixed and permeabilized using a Cytofix Kit (BD Biosciences). The flow cytometric acquisition was performed with live/dead aqua, anti-B220 eFluor450, anti-CD3 phycoerythrin (PE)-Cy7, anti–CD4-allophycocyanin (APC) Alexa Fluor 750, anti-Foxp3 PE-Cy5.5, anti-CD25 APC, anti–IL-4 PE, anti–IL-10 peridinin chlorophyll protein complex (PerCP) Cy5.5, anti–IL-17 Alexa Fluor 700, and anti–IFN-γ Alexa Fluor 488 (BD Biosciences). The cells were acquired using LSRFortessa (BD Biosciences) and analyzed by FlowJo software (TreeStar, Inc.). Cytokines and chemokines in serum were detected as described previously and measured using a multiplex sandwich-ELISA (Biorad) (4).

Statistical Analysis. Unpaired Student *t* tests were used to analyze differences of saliva flow rate (SFR), tear flow rate (TFR), immune cells in salivary gland (SG), and cytokine productions in serum. The Spearman rank correlation analysis was used to analyze the correlation of BMP6 expression with clinical parameters, including unstimulated saliva, serum IgG, C3, C4, anti-Ro/SSA, anti-La/SSB, and ANA, in patients with pSS.

For AQP5 expression comparison in BMP6 high/low-expressing patients with pSS and HVs, sample size calculations based on a two-sample *t* test with unequal variances show that $n = 7$ patients with pSS ($n = 5$ high and $n = 2$ low BMP6 expression) would be sufficient to have at least 80% power to find differences of this magnitude or greater as significant at the 5% level to detect differences of at least 140 fluorescent units between group BMP6 means, assuming variability similar to the variability observed in out preliminary data (10) (www.stat.ubc.ca/~rollin/stats/ssize/n1.html). All analyses were performed with GraphPad Prism statistical software (version 4.02; GraphPad Software, Inc.) using a P value ≤0.05 as statistically significant.

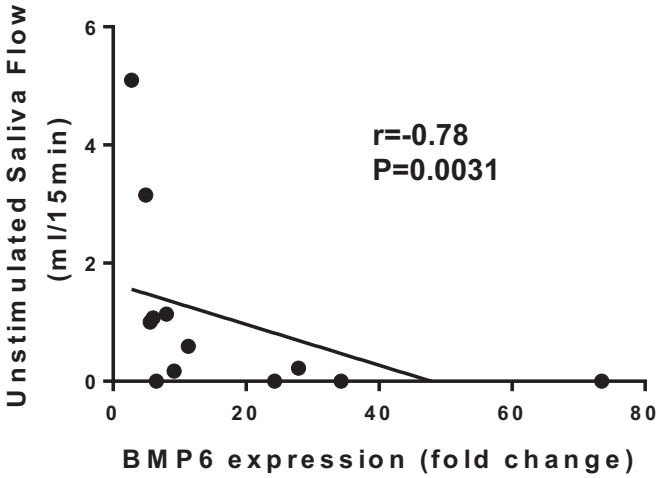

Fig. S1. Correlation analysis of BMP6 expression and unstimulated saliva output. BMP6 expression in individuals with pSS ($n = 12$) was measured by quantitative PCR relative to GAPDH [ΔΔCt (Delta Delta Ct)] and is presented as fold change over the average expression in HVs ($n = 11$). Spearman rank correlation analysis indicates there is a significant negative correlation between BMP6 and salivary fluid output in patients ($r = -0.78$, $P = 0.0031$).

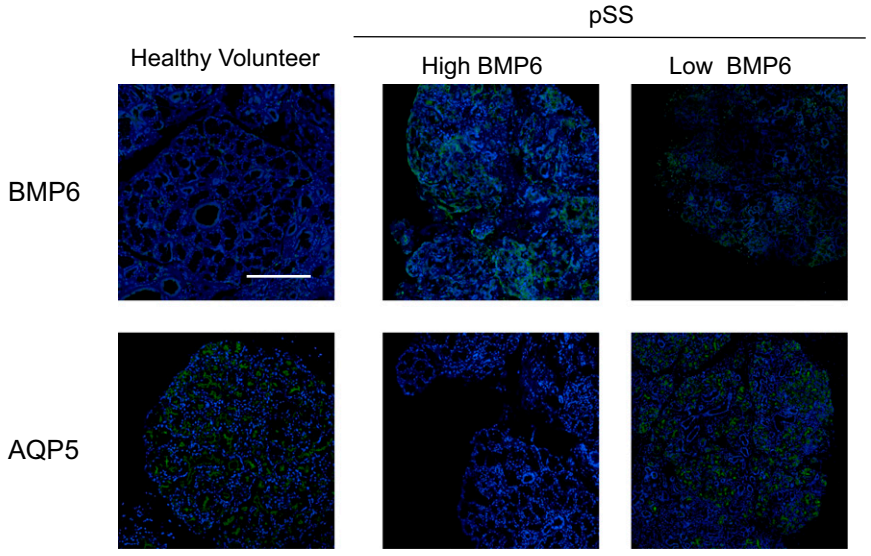

Fig. S2. BMP6 and AQP5 expression in MSGs from patients with pSS and HVs. Stitched immunofluorescence images of BMP6 and AQP5 in the entire biopsied sample area are shown. (Scale bar, 300 μm.)

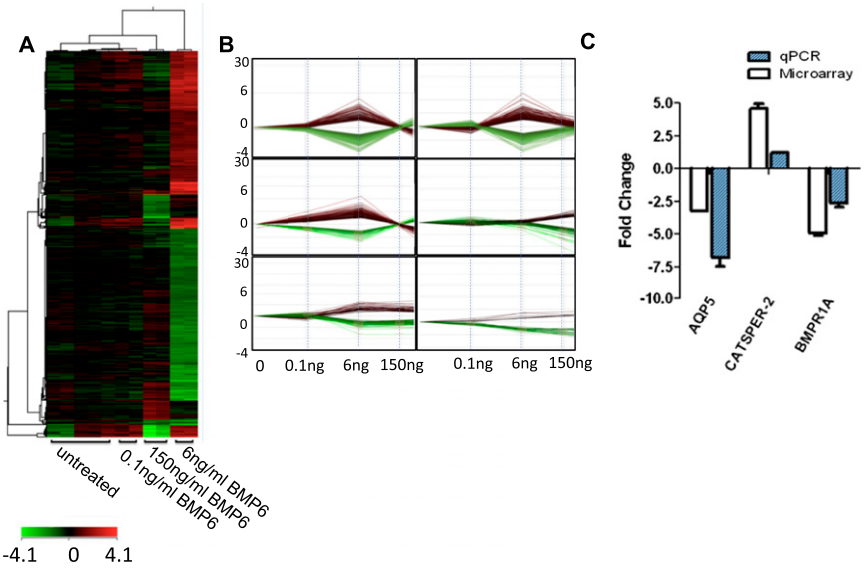

Fig. S3. BMP6 has a bimodal effect on gene expression in HSG cells. (*A*) Heat map of positive probes as well as unsupervised clustering of samples. Unsupervised clustering of treated HSG cells shows clustering of the group treated with 0.1 ng/mL with the control samples followed by the group treated with 150 ng/mL. The most significant number of gene changes was observed within the 6-ng/mL treatment group. (*B*) Bimodal effect of gene expression in HSG cells in response to treatment with increasing concentrations of BMP6 at dosages of 0.1 ng/mL, 6 ng/mL, and 150 ng/mL. Analysis of the patterns of gene expression at the different concentrations of BMP6 identified six distinct profiles. Brown and green lines represent genes following the mirror image pattern of each other. (*C*) Quantitative PCR (qPCR) detection of selected genes extracted from HSG cells after BMP6 treatment with 6 ng/mL shows agreement with the results of the microarray study on samples from patients with pSS. The results obtained using the custom microarray platform were validated by qPCR and compared with microarray data from patients with pSS, which showed a similar trend in expression. CATSPER-2, cation channel sperm associated 2.

Table S1. Characteristics of the HV control subjects and patients with pSS

Classification	pSS (n = 12)	HV (n = 11)	Normal range	P value*
Age, y; median (minimum–maximum)	50.5 (23–72)	46 (30–60)		
Sex	5M, 7F	4M, 7F		
Race, n (white/African American/Asian/other)	7/2/1/2	8/1/0/2		
Unstimulated SFR, mL over 15 min; median (minimum–maximum)	0.41 (0–5.1)	4.9 (0–13.74)		0.0200
Focus score, median (minimum–maximum)	1.5 (0–4)	0 (0–2)	0–1	0.0038
SSA-positive	9	0	0–19EU, negative	0.0003
SSB-positive	9	0	0–19EU, negative	0.0003
ANA-positive	10	1	≤1EU, negative	0.0006
C3 complement, mg/dL; mean ± SD	106 ± 27	130 ± 25	69–75	0.0509
C4 complement, mg/dL; mean ± SD	19 ± 8	26 ± 23	10–40	0.0314
IgG, mg/dL; mean ± SD	1,607 ± 467	1,165 ± 282	642–1,730	0.0165

Except where indicated, values are the number of subjects. All patients had a diagnosis of pSS according to the American-European Consensus Group Criteria. EU, ELISA unit; F, female; M, male.

*Statistical analysis methods: For unstimulated saliva, C3, and IgG comparison, an unpaired Student t test was used. For focus score, a Mann–Whitney U test was used. For SSA, SSB, and ANA comparison in two groups, a Fisher's exact test was used. Data were analyzed using Prism 5.

Table S2. Correlative analysis of gene expression changes in the BMP6-treated HSG and mice

Symbol	Entrez gene name	0.1-ng BMP6 fold change	6-ng BMP6 fold change	150-ng BMP6 fold change	BMP6 mice fold change	PCC
AQP5	Aquaporin 5	−1.458	−7.156	ND	−1.896	0.98
EGR1	Early growth response 1	2.049	4.211	ND	1.16	−0.89
TNFRSF1B	TNF receptor superfamily	−1.721	−2.468	ND	−1.265	0.85
IGSF10	IgG superfamily, member 10	−1.388	−2.58	ND	−1.216	0.83
COX7B	Cytochrome c oxidase subunit VIIb	1.299	1.747	ND	1.278	−0.62
NKRF	NFKB repressing factor	1.469	1.913	ND	1.342	−0.62

ND, not detected; PCC, Pearson correlation coefficient.

Table S3. Flow Cytometry Assay of Salivary Gland $CD4^+$ Immune Cells from AAV2-GFP and AQP1 mice

$CD4^+$ cells	GFP	AQP1	*P* value
$CD3^+$	9.2 ± 0.8	4.9 ± 1.1	0.046
IFN^+	11.4 ± 3.7	3.5 ± 0.7	0.052
IL-4^+	2.41 ± 0.5	0.6 ± 0.3	0.047
IL-17^+	9.2 ± 0.3	5.3 ± 0.4	0.004

Salivary gland lymphocytes were collected from AAV2-mediated GFP and AQP1 overexpression C57BL/6.NOD*Aec1*/*Aec2* mice and then detected for CD3, CD4, IFN-γ, IL-4, and IL-17 expression by flow cytometry. Data shown are the mean ± SEM for each animal group (AAV2-GFP and AAV2-AQP1, respectively). An unpaired Student's *t* test was used to analyze differences of expression.