SUPPORTING INFORMATION

Increased expression of the epithelial anion transporter Pendrin/SLC26A4 in nasal polyps of patients with Chronic Rhinosinusitis

Sudarshan Seshadri, PhD[1*], Xiang Lu, MD.PhD[1,2*], Matthew R. Purkey, BS[1], Tetsuya Homma, MD[1], Andrew Wonho Choi, BS[1], Roderick Carter, BS[1], Lydia Suh, BSc[1], James Norton, MS[1], Kathleen E. Harris, MS[1], David B. Conley, MD[3], Atsushi Kato, PhD[1], Pedro C. Avila, MD[1], Barbara Czarnocka, PhD[4], Peter A Kopp, MD[5], Anju T Peters, MD[1], Leslie C Grammer, MD[1], Rakesh K. Chandra, MD[3], Bruce K. Tan, MD[3], Zheng Liu, MD[2], Robert C. Kern, MD[3], Robert P. Schleimer, PhD[1,3]

[1]Division of Allergy and Immunology, Northwestern University Feinberg School of Medicine, Chicago, IL, USA

[2] Department of Otolaryngology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, PR China

[3]Department of Otolaryngology—Head and Neck Surgery, Northwestern University Feinberg School of Medicine, Chicago, IL, USA

[4] Department of Biochemistry and Molecular Biology, The Centre of Postgraduate Medical Education, Marymoncka 99/103, Warsaw 01-813, Poland

[5]Division of Endocrinology, Metabolism, and Molecular Medicine, Northwestern University Feinberg School of Medicine, Chicago, IL, USA

* These authors contributed equally.

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Address correspondence to Dr. Robert P. Schleimer, Ph.D.,

Division of Allergy and Immunology, Northwestern University Feinberg School of Medicine,

240 E Huron St., Mcgaw- Rm #M-318, Chicago, IL 60611 USA.

Phone: 312-503-0076; FAX: 312-503-0078; E-mail: rpschleimer@northwestern.edu

METHODS

Cell Culture

NECs were collected during surgery and cultured under air-liquid interface (ALI) conditions[1]. NECs were first plated in a single well of a 6-well culture plate coated with collagen (Vitrogen; Collagen Biomaterials, Palo Alto, CA) for expansion. After reaching confluence, cells were trypsinized and plated in 0.4μm pore membrane inserts of a 12-well transwell plate (Costar, Corning, NY). Cells were grown in Bronchial Epithelial Growth media (BEGM) (Lonza) with media on apical and basal side until they reached confluence. Confluent cells were then cultured under ALI conditions. Apical media was removed and the basal media was replaced with ALI media containing BEBM/Dulbecco's modified Eagle's medium (DMEM) supplemented with BEGM SingleQuot kit (Lonza) and additional bovine serum albumin (1.5 μg/ml) and retinoic acid (5×10^{-8} M) (Sigma-Aldrich (St. Louis, MO)) to induce differentiation. The ALI medium was changed 3 times a week for 3 weeks. After 21 days, cells were changed to ALI media without hydrocortisone for 48h and then used for experiments. For stimulation with cytokines, ALI media with cytokines were added apically (50 μl) and basally (950 μl) and cells were stimulated for 24h for experiments evaluating RNA and 48h for experiments evaluating protein. In some experiments, to mimic chronic cytokine exposure, NECs were stimulated with cytokines for one week. Media containing cytokines was changed on days 2 and 4 and the cells were harvested on day 7 for assessment of RNA and protein. For dexamethasone experiments, cells were first pretreated with dexamethasone or DMSO for 1h and then stimulated with cytokines as indicated for further 24h. Cells were then harvested in RNA lysis buffer containing β-mercaptoethanol.

Real-time PCR

Total RNA from sinonasal tissue was extracted using QIAzol (Qiagen, Valencia, CA) using RNeasy purification system according to manufacturer's instructions. NECs

(scrapings and ALI cultured) were lysed in RNA lysis buffer supplemented with β-mercaptoethanol and RNA was extracted according to the manufacturer's protocol (Clontech, Mountain View, CA). DNA contamination was eliminated by treatment of RNA with DNase I during purification. Good quality RNA from scraping cells, as determined using a agilent bioanalyzer (Agilent Technologies, Santa Clara, CA), was used for real-time PCR. One microgram of RNA was converted to single-strand cDNA using SuperScript II reverse transcriptase system (Invitrogen, Carlsbad, CA) using random primers. Semi-quantitative real-time RT-PCR was performed with a TaqMan method using an Applied Biosystems 7500 Sequence Detection System (Applied Biosystems, Foster City, CA) in 15 µl reactions (7.5 µl of 2x TaqMan Gene Expression Master mix (Applied Biosystems), 0.75 µl of 20x primer/probe mix and 5 µl of 2 ng/µl cDNA). All primers/probes were purchased from Applied Biosystems. Median expression of GUSB (Human ß-glucuronidase endogenous control, PN; 4326320E) was used for normalizing gene expression. Exact copy number of the target genes was determined by running serial dilutions of quantified PCR product of the target gene as standards in the PCR reaction. Target gene expression was expressed as copies/ng of total RNA. In some experiments, the delta-CT method was used to assess gene expression.

Extraction of proteins from sinus tissue and cultured epithelial cells

Tissue proteins were extracted as described in Peters, A.T. et al [2]. Briefly, tissues were cut into small pieces and resuspended in PBS containing 0.05% tween 20 supplemented with a protease inhibitor cocktail (Sigma). Tissues were lysed in a Bullet Blender (Next Advance) with 1.6 mm

stainless steel beads for 4 minutes twice at setting 7. Tissue lysates were centrifuged at 1968 x g to clear the debris. Tissue lysates were stored at -20ºC until further use. Proteins from cultured cells were extracted using RIPA buffer (50 mM Tris pH 7.4, 1% SDS, 1mM EDTA, 150 mM NaCl, 0.25% Sodium deoxycholate with protease and phosphatase inhibitors). Protein concentrations in all samples were determined using a BCA assay (Thermoscientific) following the manufacturer's protocol. Proteins in the preparation were resolved on 4-12% Bis-Tris gel (Life Technologies, Carlsbad, CA), and transferred to a polyvinyl difluoride membrane for immunoblotting. Membranes were incubated with affinity purified pendrin PP1 (1.0 µg/ml) and actin antibody (1:10,000; MP Biomedicals, clone C4). The PP1 antibody is directed against the first 15 amino acids at the N-terminal of the protein. This antibody has been previously described in detail and has been used by other groups to characterize pendrin[3, 4]. After washing, blots were incubated with appropriate secondary antibody (Li-Cor Biosciences, Lincoln, NE). To quantify pendrin protein in tissue extracts, immunoblots were digitalized and quantified with Odyssey imaging software (Li-Cor Biosciences, Lincoln, NE). Densitometry values for pendrin were normalized to those of the housekeeping gene β-actin.

Immunohistochemistry

Immunohistochemistry was performed as described in Peters, A.T. et al [2]. Briefly, nasal tissue was dehydrated, infiltrated, and embedded with paraffin, and tissue was sectioned at 3 µm by using a Leica RM2245 Cryostat (Leica Microsystems Inc.; Bannockburn, IL). All sections were de-paraffinized and rehydrated; tissue sections were boiled for 20 minutes in citrate-based buffer for antigen unmasking (pH 6.0; Vector Laboratories; Burlingame, CA). All sections were then blocked for endogenous peroxidase activity with 3% H_2O_2/methanol. After rinsing, sections were treated to block nonspecific binding with 5% goat serum/0.3% Tween-20/PBS. Tissue sections were then incubated overnight at 4°C with 0.03 µg/ml of rabbit anti-human pendrin antibody or

control rabbit IgG. Sections were rinsed and then incubated with biotinylated secondary goat anti-rabbit antibody (Vector Laboratories) at a 1:500 dilution for 1 hour at room temperature. After another rinse, sections were incubated in ABC reagent (avidin–biotin–horseradish peroxidase complex, Vector Laboratories) for 1 hour at room temperature. Sections were rinsed again and incubated in diaminobenzidine (DAB) reagent (Invitrogen) for 10 minutes at room temperature. They were then rinsed in deionized H_2O, counterstained with hematoxylin, dehydrated, cleared, mounted, and coverslipped by using Cytoseal 60 (Richard-Allan Scientific; Kalamazoo, MI) in preparation for microscopic analysis.

Sections were observed using an Olympus IX71® Inverted Microscope at a magnification of 200X and a MicroFire AR digital microscope camera (Optronics; Goleta, CA).

FIGURE LEGENDS

Figure E1.

Pendrin peptide blocked detection of pendrin by pendrin specific antibody in sinonasal tissues.

Total protein (30 μg) from sinonasal tissues and recombinant pendrin were separated by gel electrophoresis. Proteins were transferred to PVDF membrane. Membranes were then incubated with pendrin antibody premixed with PBS or pendrin peptide overnight. Blots were developed following normal immunoblot protocol. Blot shown is representative from at least 2 experiments using different sinonasal tissues from multiple donors.

Figure E2.

A trend towards increased pendrin expression in polyp epithelial cells compared to uncinate control epithelial cells.

Nasal Scraping epithelial cells (NECs) from control, CRSsNP, CRSwNP (uncinates) and polyps were obtained during surgery. RNA was isolated and the expression of pendrin in NECs was analyzed using real-time PCR.

Figure E3.

Periostin expression was increased in nasal polyps of patients with CRS and periostin expression correlated with IL-13 expression in sinonasal tissues.

Expression of periostin in sinonasal tissues was analyzed by real-time PCR (**A**). The relationship between expression of periostin and IL-13 in sinonasal tissues was analyzed using a Spearman Rank Correlation (**B**). Data for IL-13 was used from figure 4. * $p<0.05$, ***$p<0.001$

Figure E4.

Expression of pendrin mRNA was increased by Th2 cytokines and IL-17 stimulation in a dose dependent manner in differentiated airway epithelial cells.

Differentiated nasal epithelial cells (NECs) were untreated (CTL) or stimulated with increasing concentrations (0.1 -50 ng/ml) of cytokines as indicated for 24 h. Pendrin gene expression was quantified using real-time PCR (n=3-12). Gene expression was normalized to actin and expressed as fold change over untreated control samples.

Figure E5.

Expression of periostin was not synergistically induced by combined treatment of Th2 and IL-17A in differentiated airway epithelial cells.

Real-time PCR analysis of periostin expression in differentiated NECs stimulated with cytokines alone (10 ng/ml) or in combination (10 ng each) as indicated for 24 h. Shown is data from 6 experiments.

Figure E6.

Expression of pendrin was additively induced by combined treatment with IL-13 and IL-1β in differentiated airway epithelial cells.

Real-time PCR analysis of pendrin expression in differentiated NECs stimulated with cytokines alone (10 ng/ml) or in combination (10 ng each) as indicated for 24 h. Shown is data from 2 experiments.

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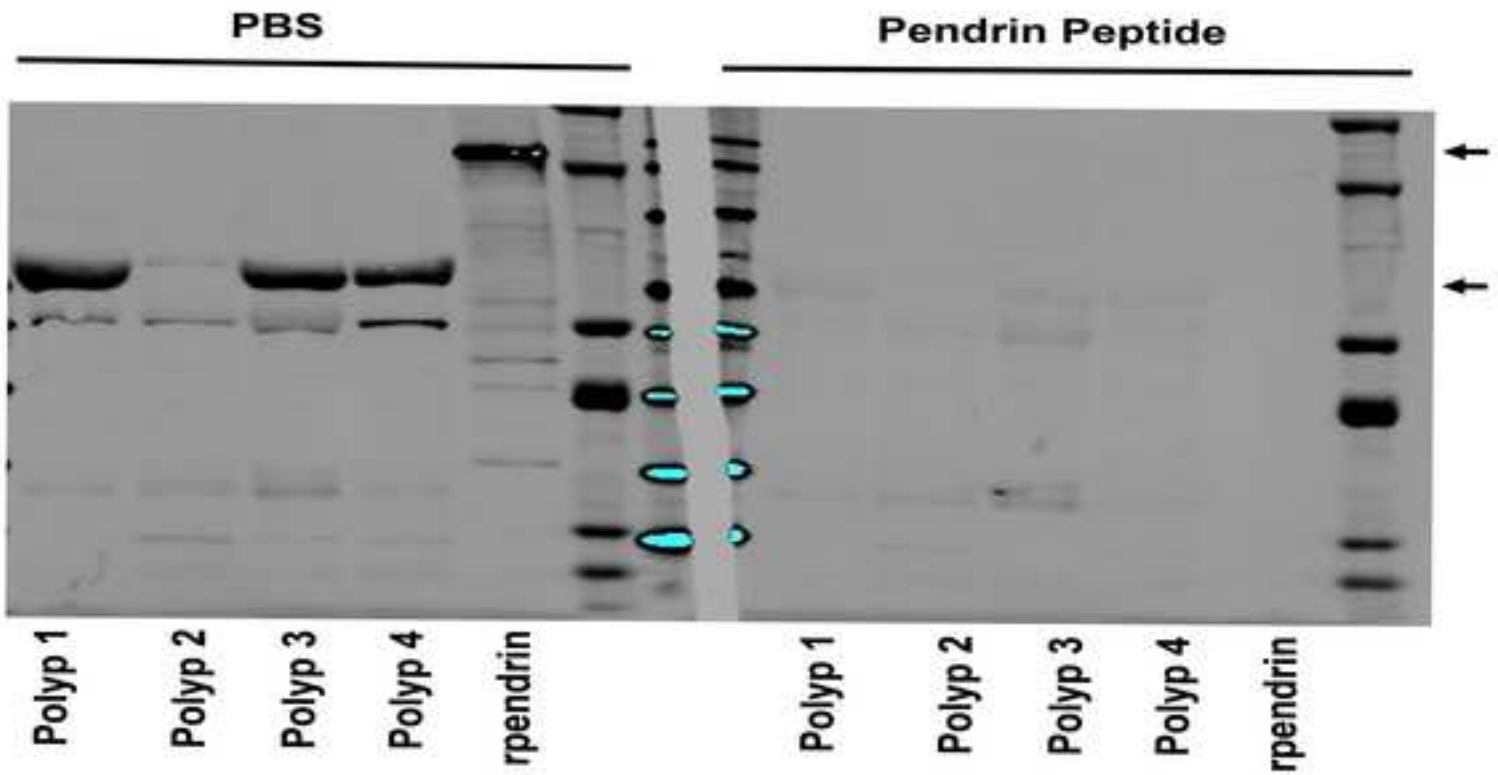
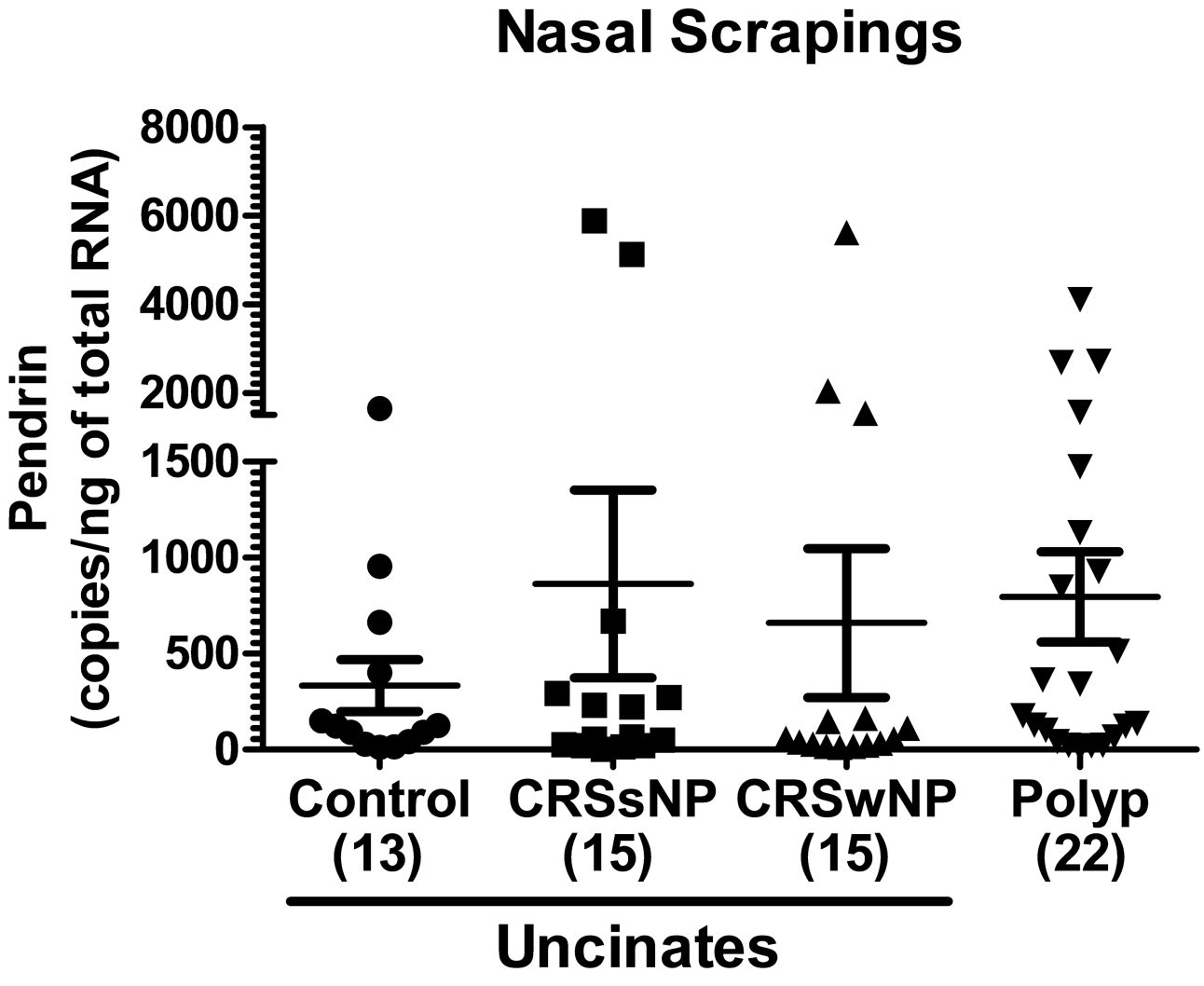


Seshadri Fig. E1



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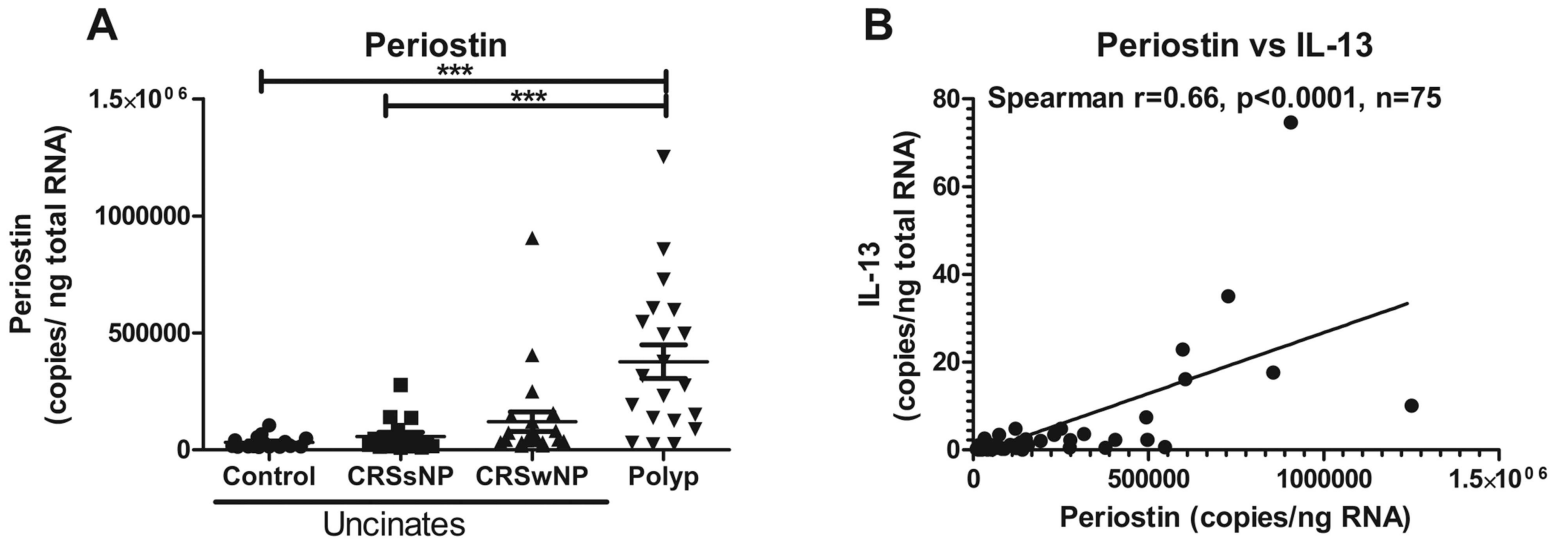

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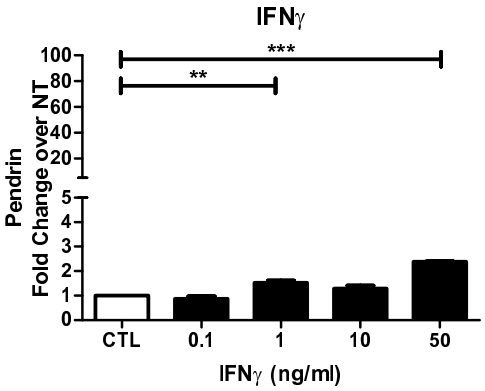

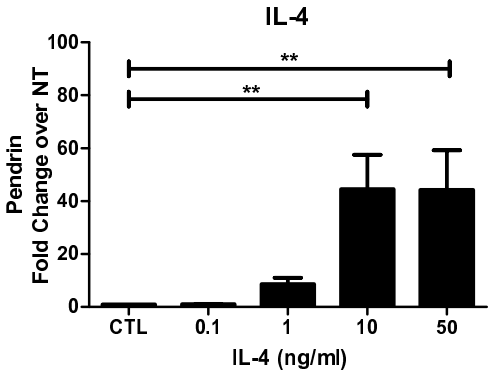

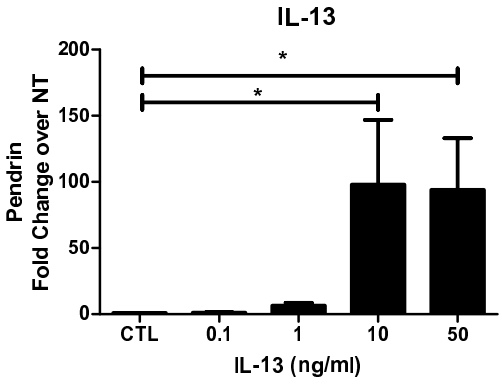

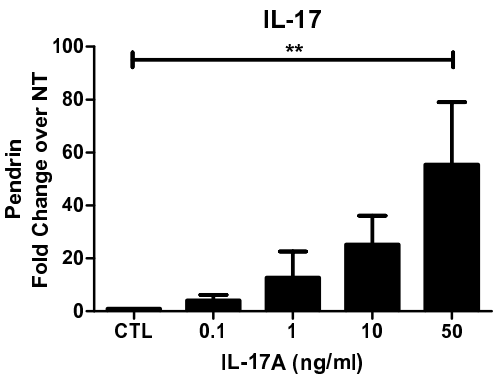

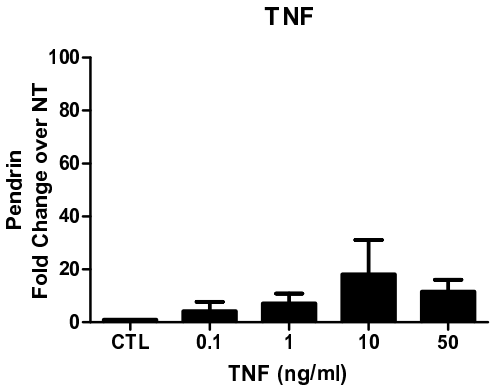

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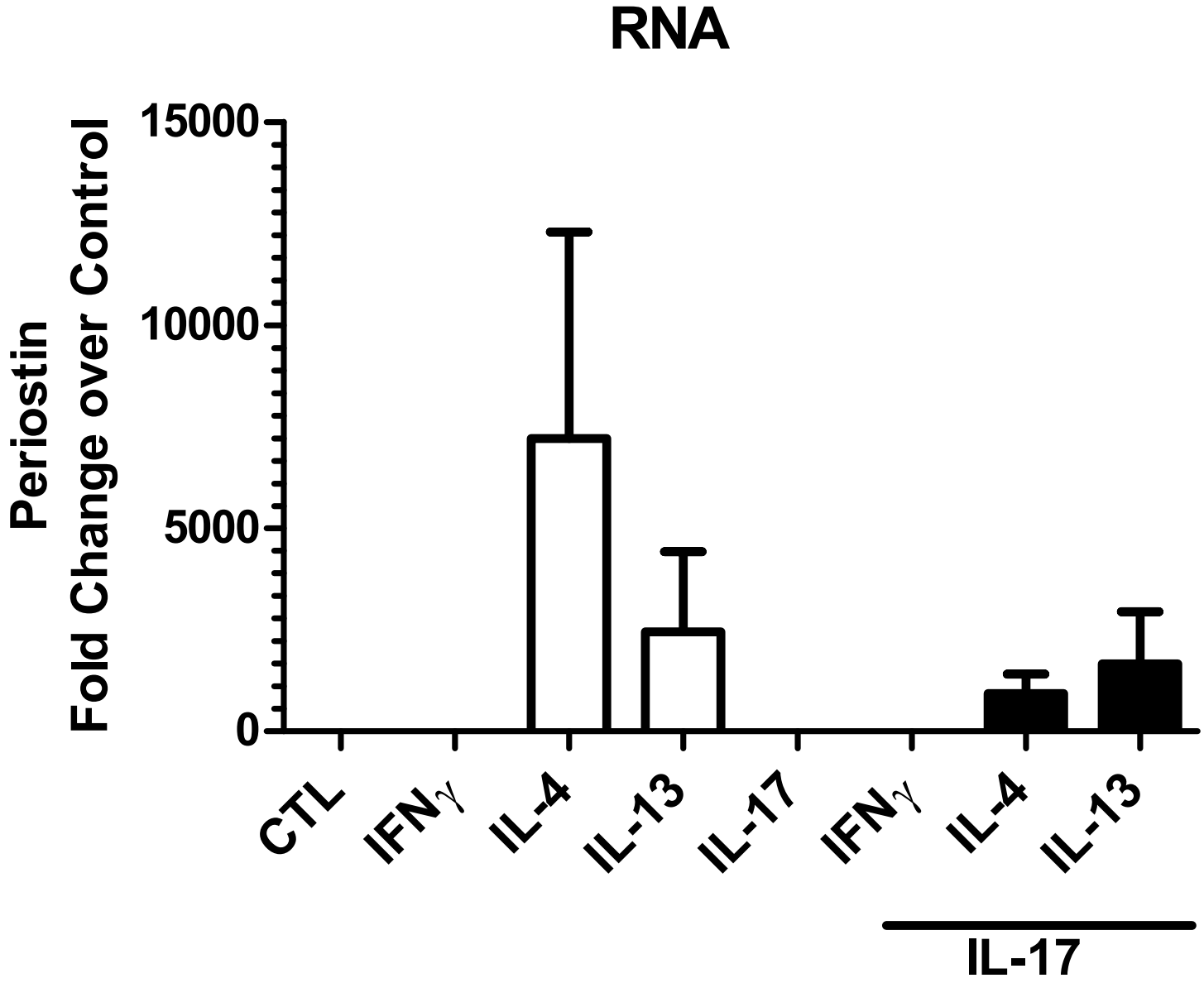

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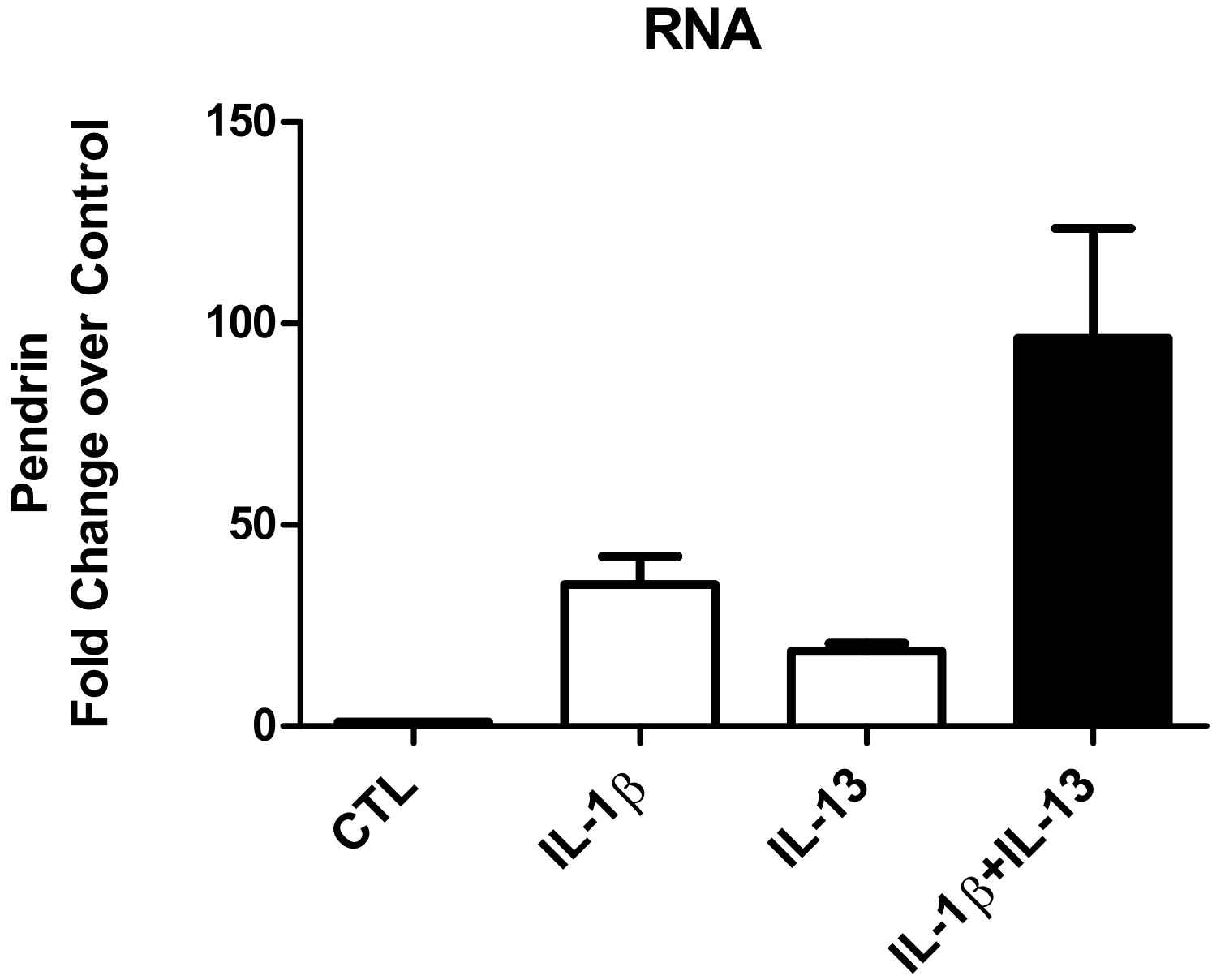

Seshadri Fig. E6