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SUPPORTING INFORMATION

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

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EMSY is increased and activates TSLP & CCL5 expression in eosinophilic esophagitis

To the Editor:

Eosinophilic esophagitis (EoE) is a clinicopathologic disease characterized by an increased number of eosinophils (greater than or equal to 15 eosinophils per high powered field) in the esophageal mucosa, likely due to an immune or antigenic response.¹ Specifically, there is an increased production of esophageal epithelial-derived pro-inflammatory mediators such as thymic stromal lymphopoietin (TSLP), eotaxin 3 (CCL26), RANTES (CCL5), calpain 14 (CAPN14), and interleukin (IL)-33,² which ultimately results in eosinophilic infiltration of the esophageal epithelium. Thymic stromal lymphopoietin is mainly secreted by epithelial cells and is a IL-7 like cytokine that is able to induce a strong T helper type 2 (Th2) inflammatory response.³ Thymic stromal lymphopoietin has been shown to play a crucial role in EoE pathogenesis.³ Similarly, IL-33 is an epithelial-derived Th2 cell agonist and allergenic ligand that has been associated with EoE.⁴ Chemokine (C-C motif) ligand 5 (CCL5) is a cytokine that is known to contribute to several atopic and inflammatory eosinophilic diseases, including EoE.

Genomewide association studies (GWAS) data from our group show a strong association between EoE and a novel gene locus c11orf30-EMSY, whose role is unknown in esophageal cells.⁵

Initially, EMSY was described as a transcriptional factor able to interact with and inhibit the function of BRCA2, a known tumor suppressor gene in mammary cancer.⁶ In mammary and ovarian cancer cells, Akt1 phosphorylates EMSY at Ser209, which releases EMSY-mediated transcriptional repression of interferon stimulating genes.⁷ More recently, EMSY has been shown to regulate post-translational modifications of core histones to induce gene expression⁸ of its target genes and stimulate cell proliferation. Furthermore, Amaral et al proposed that the locus c11orf30 is associated with atopy.⁹ In addition to mammary epithelial cells, EMSY expression has also been demonstrated in multiple types of epithelium including esophageal, ovarian, kidney, and bronchial epithelium as well as cells of hematopoietic lineages.^{6,8,10} As the esophageal epithelium plays a central role in the pathogenesis of eosinophilic esophagitis, we set out to determine the tissue-specific role of EMSY in esophageal epithelium and its potential role in regulating the expression of key epithelial cytokines and chemokines.

The aim of this study was to investigate the role of EMSY in esophageal epithelial cell biology and in EoE and to determine if EMSY is involved in gene regulation in the esophageal epithelium. To investigate the function of EMSY in normal esophageal epithelium,

we inhibited EMSY by siRNA transfection and then determined the effects on expression of epithelial-derived mediators such as CAPN14, IL-33, CCL5, and TSLP. To also determine a possible pathogenic role of EMSY in EoE, we measured the RNA levels of EMSY in patients with EoE and controls.

EPC2-hTERT cells, which are immortalized non-transformed human esophageal epithelial cells, and 293T immortalized human kidney epithelial cells were grown in monolayers at 37°C in a humidified 5% CO₂ incubator. The EPC2-hTERT cells were cultured in keratinocyte serum-free medium (KSFM) supplemented with epidermal growth factor, bovine pituitary extract, penicillin, and streptomycin.

EPC2-hTERT cells were resuspended in lysis buffer. The proteins were separated by electrophoresis using NuPAGE Tris-Acetate Mini gels (Life Technologies, Carlsbad, CA) and then transferred to nitrocellulose membranes. The membranes were blocked overnight at 4°C with 5% non-fat dry milk plus 0.1% Tween 20 in 20 mM Tris-buffered saline. The membranes were then bathed in diluted primary EMSY goat antibody (Abcam, Cambridge, MA) followed by secondary horseradish peroxidase (HRP)-labeled anti-goat IgG antibody (MBL International Corporation, Woburn, MA). Immobilon Western chemiluminescent HRP detection kit was used for chemiluminescent protein signal detection (Millipore, Billerica, MA).

EPC2-hTERT cells were seeded into 6-well tissue culture plates containing 20 nM of either FlexiTube Gene Solution EMSY siRNA or "All Stars" Negative Control siRNA (Qiagen, Valencia, CA) diluted in Opti-Minimal Essential Medium 1 (Gibco, Life Technologies, Carlsbad, CA). 5×10^5 cells per well were mixed with Lipofectamine RNAiMAX. KSFM growth media without antibiotics were added, and the plates were incubated at 37°C for 48 hours. RNA was isolated according to the manufacturer's protocol (Qiagen RNeasy Maxi Prep kit, Valencia, CA), and concentrations were determined using an Eppendorf BioPhotometer plus and a NanoDrop device. RNA isolated in all experiments had acceptable purity levels. cDNA was then synthesized by following the manufacturer's random primer protocol from the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, California).

The transfection experiments were repeated with either "All Stars" Negative Control siRNA or EMSY siRNA, both tagged with Alexa-Fluor (AF) 488. All cells were sorted with a FACSJazz electrostatic droplet sorter gating on all AF488⁺ cells for subsequent RT-PCR and Western blot analysis. Non-transfected EPC2-hTERT cells were used as a negative gating control.

TaqMan Universal PCR Master Mix and Gene Expression Assays (Applied Biosystems, Foster City, CA) were used for the following genes: EMSY, TSLP, CAPN14, IL33, CCL5, and the endogenous control Ras homolog family member A (RhoA). All reactions were run in triplicate on 96-well optical plates on an Applied Biosystems 7500 Fast Real-Time PCR System device, using the ddCT mode. The data were calibrated to "All Stars" Negative Control siRNA. The average Ct count for each of the genes was between 20 and 30.

This study was approved by the Institutional Review Board of The Children's Hospital of Philadelphia. Following the 2011 EoE

clinical guidelines, active EoE was defined as 15 or more eosinophils per high powered field (eos/hpf) on esophageal biopsy after treatment with a proton pump inhibitor for greater than 4 weeks. Active EoE patients ($n = 9$) had a median eosinophil count of 35 eos/hpf (interquartile range 30-50, min 20 max 100 eos/hpf). Patients with a preexisting diagnosis of EoE that had less than 5 eos per hpf were termed inactive EoE ($n = 5$). Control patients had zero eos/hpf ($n = 10$). Figure 3A depicts patient demographics. Patients were excluded for systemic use of immunosuppressants or other significant gastrointestinal comorbidities such as inflammatory bowel disease.

RNA was isolated from esophageal biopsies of control or EoE patients (mirVana, Ambion, Foster City, CA). nCounter Nanostring technology quantifies RNA transcripts via hybridization of the capture and detection probe to RNA with multiplexed detection. 100 ng of total RNA was hybridized to the probe, and samples were analyzed in the nCounter system according to the manufacturer's instructions. Analysis was performed in nSolver 3.0. Samples with quality control or normalization anomalies were excluded.

RT-PCR results are shown as a mean with error bars displaying the standard error of the mean. Two-tailed Student's *t* test and a nonparametric two-tailed Mann-Whitney test were used to determine statistical significance. Results were considered to be statistically significant at $P < \text{or} = .05$.

EPC2-hTERT cells were used as an in vitro model to investigate the role of EMSY in normal esophageal epithelial cell physiology. These cells are non-transformed and were immortalized by constitutively active telomerase without genetic abnormalities. Therefore, they are used as a reliable model for in vitro analysis of normal esophageal epithelial function.¹¹ Using this model, for the first time, we confirmed that EMSY protein is in fact expressed in esophageal epithelial cells (Figure 1). Indeed, Western blot analysis of EPC2-hTERT cells showed that EMSY protein is present at comparable levels to 293T kidney epithelial cells, which are known to express EMSY (data not shown).

Transfection of EPC2-hTERT cells with EMSY siRNA was successfully achieved and resulted in an average 61% reduction in EMSY gene expression ($P < .0003$) both at the protein (Figure 1) and RNA level (Figure 2). Using EMSY siRNA tagged with Alexa-Fluor 488, we demonstrated that 97% of the cells were successfully transfected with the EMSY siRNA or negative control siRNA. (data not shown).

To determine if EMSY is involved in regulation of the cytokines TSLP, CAPN14, IL-33, or CCL5, we repressed EMSY gene expression via siRNA transfection in esophageal epithelial cells and measured the mRNA expression levels of the various genes. Transfection with EMSY siRNA led to an average 41% reduction in TSLP expression ($P < .003$) and a 77% reduction in CCL5 expression ($P < .00003$) compared with negative controls (Figure 2). There was no significant change in the expression of CAPN14 or IL33.

EMSY levels in fresh biopsies of EoE patients have not previously been examined. Using digital multiplex gene expression, we

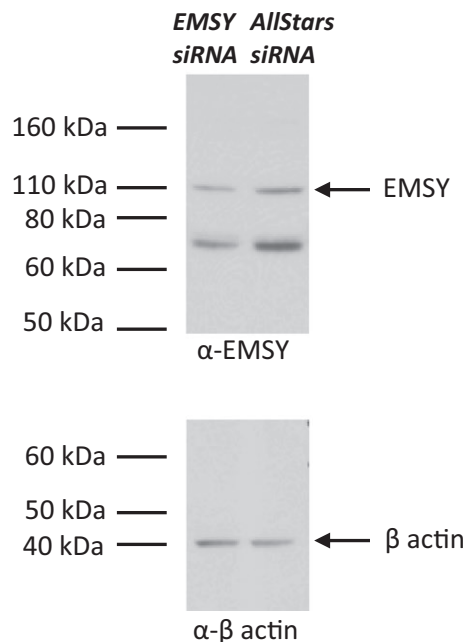


FIGURE 1 Decreased EMSY protein expression. Top: Western blot analysis of EMSY protein levels after Lipofectamine RNAiMAX transfection of EPC2-hTERT cells with siRNA targeting EMSY mRNA (lane 1) or All Stars negative control siRNA with no homology to any known mammalian gene (lane 2). Bottom: Western blot of identical PVDF membrane from top stripped and reprobed with β-actin endogenous control. Protein reduction was calculated using ImageJ software, comparing pixel intensity of EMSY band divided by pixel intensity of β-actin band between EMSY and control siRNA. This experiment was replicated in triplicate

measured EMSY RNA in 9 pediatric patients with active EoE, 5 with inactive EoE, and 10 age-matched healthy controls (Figure 3A), and we observed that EMSY was upregulated in biopsies of patients with active EoE compared with control patients and EoE patients with quiescent disease (Figure 3B).

Polymorphisms in EMSY have been associated with several allergic and immunologic conditions including atopic dermatitis (AD), asthma, allergic rhinitis, inflammatory bowel disease, and food allergy.¹² Our group previously showed in a GWAS that EMSY is among three novel loci associated with EoE.⁵ Additional data suggest that there is an association between polymorphisms in EMSY and allergen polysensitization.⁹ Further studies could be useful to determine whether polymorphisms in EMSY can be used to predict EoE patients who are more atopic than others. The precise role of EMSY in regulation of atopic inflammation and esophageal epithelial biology is unknown.

In this study, we demonstrate for the first time that EMSY RNA and protein are expressed in the immortalized non-transformed human esophageal epithelial cell line EPC2-hTERT. We decreased EMSY gene expression using siRNA which led to decreased protein expression. Our novel data reveal that EMSY gene repression resulted in decreased TSLP and CCL5 expressions, without significant changes in IL33 and CAPN14 expressions. This has clinical relevance in EoE, as our in vivo experiments show that EMSY is significantly

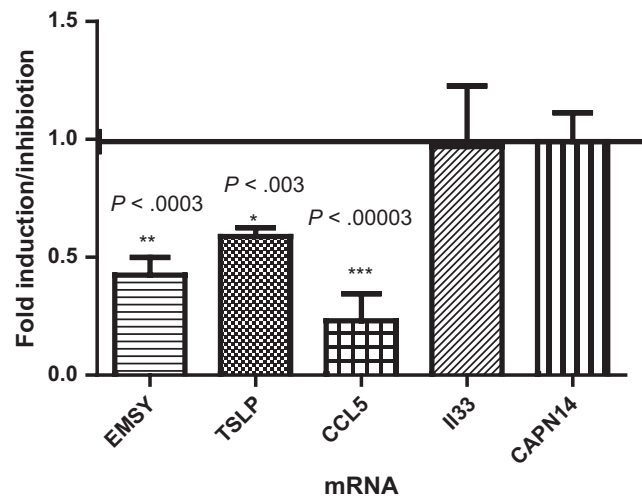


FIGURE 2 Changes in gene expression. Transfection of EPC2-hTERT cells with EMSY siRNA resulted in a reduction in gene expression of TSLP, CCL5, and EMSY, and no significant change in the expression of IL33 and CAPN14. Data are average of 9 experiments. $P < .05$ is considered statistically significant (using 2-tailed Student's *t* test and a nonparametric two-tailed Mann-Whitney test)

(A)

	Active EoE n = 9	Inactive EoE n = 5	Control n = 10
Mean age (years; interquartile range)	12; 8-13	10.5; 8-14.75	12; 7-15.5
Male (%)	77	83	60

(B)

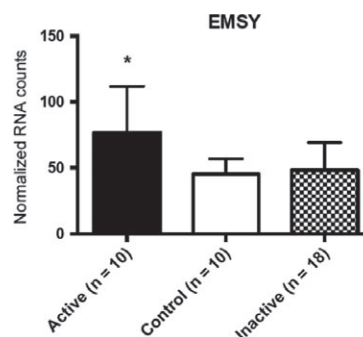


FIGURE 3 EMSY expression in active EoE, inactive EoE, and control patients. A, Patient demographics. B, EMSY gene expression is significantly upregulated in those patients with active EoE compared to patients with inactive EoE and controls (*denotes $P < .05$, ANOVA with Tukey multiple comparison test)

increased in active EoE, supporting the findings of our in vitro experiments that EMSY is an activator of TSLP and CCL5 expression.

Thymic stromal lymphopoietin is an inflammatory cytokine that has been shown to play a critical role in EoE by both genetic and molecular studies.^{5,11} For the first time, we provide evidence that EMSY has a role in modulating expression of TSLP. Our results

suggest that EMSY is upregulated in esophageal biopsies of children with active disease and positively regulates TSLP in esophageal epithelial cells.

Furthermore, our data show that the inflammatory chemokine CCL5 (previously known as RANTES) is also regulated by EMSY, and EMSY therefore may be a target for both TSLP and CCL5 inhibition.

EMSY has been shown to be able to repress or activate gene transcription.^{7,8} In mammary and ovarian cancer cells, it has been demonstrated that Akt1 phosphorylates EMSY at Ser209, which releases EMSY-mediated transcriptional repression of interferon stimulating genes.⁷ On the other hand, EMSY has been shown to regulate post-translational chromatin modifications such as histone methylation.⁸ Specifically, EMSY appears to associate with H3K4me3 at active promoters. In this way, EMSY expression is positively correlated with expression of its target genes and stimulates cell proliferation; however, further investigation is needed to determine the exact mechanism by which EMSY regulates TSLP and CCL5. EMSY has been shown to be associated with the KDM5A complex, a lysine demethylase, and facilitates KDM5A complex binding to H3K4me3-marked genes to induce its demethylation.⁸ We speculate that EMSY may modify TSLP gene expression by inducing changes in histone methylation. A limitation of this study is that we did not investigate whether silencing EMSY protein results in changes in TSLP and CCL5 protein expression.

Our study is the first to show that EMSY may be activated in EoE to induce TSLP and CCL5 gene expression in the esophageal epithelium. Additional studies are needed to confirm the role of the EMSY protein in histone methylation and post-translation epigenetic regulation of the TSLP and CCL5 genes.

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CONFLICT OF INTERESTS

The authors have no conflict of interests to disclose.

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