

TGF- β 1-induced phospholamban expression alters esophageal smooth muscle cell contraction in patients with eosinophilic esophagitis

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Background: Eosinophilic esophagitis (EoE) is a chronic antigen-mediated disease characterized by esophageal eosinophilia, remodeling, and fibrosis. TGF- β 1 is a central regulator of EoE remodeling and increases esophageal smooth muscle (ESM) cell contraction.

Objective: In this study we aimed to understand the molecular mechanisms by which TGF- β 1 could induce ESM cell contraction.

Methods: We used primary human ESM cells and esophageal myofibroblasts (EMFs) to assess the mechanisms of TGF- β 1-induced contraction. We analyzed the expression, phosphorylation, and function of phospholamban (PLN), a sarcoendoplasmic reticulum regulatory protein induced by TGF- β 1. Expression of PLN, phospho-PLN, and its regulatory pathway was analyzed in the ESM of biopsy specimens from patients with EoE and control subjects. Gene silencing in EMFs from patients with EoE was used to understand the role of PLN in contraction.

Results: TGF- β 1 induced and phosphorylated PLN in primary human ESM cells and EMFs from patients with EoE. PLN and phospho-PLN levels were increased in smooth muscle from patients with EoE compared with that seen in smooth muscle from control subjects *in vivo*. PLN inhibition significantly

diminished TGF- β 1-induced EMF contraction in patients with EoE. PLN expression and ESM/EMF contraction depended on TGF- β receptor I signals.

Conclusion: We describe a previously unrecognized mechanism for ESM cell contraction that depends on TGF- β 1, its receptors, and PLN. Because PLN levels are increased in smooth muscle from patients with EoE and PLN silencing diminishes contraction, we provide a novel potential mechanistic framework and therapeutic target for ESM dysfunction in patients with EoE. (J Allergy Clin Immunol 2014;■■■:■■■-■■■.)

Key words: Remodeling, esophagus, eosinophils, smooth muscle, TGF- β , phospholamban

Eosinophilic esophagitis (EoE) is a chronic antigen-mediated clinicopathologic disease of increasing worldwide prevalence.¹ Transmural remodeling changes, including epithelial basal zone hyperplasia, subepithelial fibrosis, and smooth muscle hypertrophy, promote disease symptoms and complications, such as esophageal narrowing, rigidity, strictures, and dysmotility.²⁻¹⁶ Dysphagia and food impactions are cardinal EoE features that can occur in the absence of esophageal narrowing or stricture,^{1,17,18} suggesting that additional mechanisms beyond fibrostenosis contribute to EoE pathogenesis.¹⁴ For example, pediatric patients with EoE have dysphagia and impactions but rarely have esophageal narrowing.^{1,17} Patterns of dysmotility in patient with EoE include increased contraction amplitude, nutcracker esophagus, achalasia, and ineffective uncoupled contractions.^{13,15,16,19-22} However, despite these multiple clinical observations, the molecular mechanisms driving esophageal smooth muscle (ESM) remodeling and dysfunction in patients with EoE remain largely undefined.

EoE remodeling depends on inflammatory cell infiltration,^{2,10,11,23,24} and TGF- β 1 levels are significantly increased in the esophagi of patients with EoE.^{2,23,25-27} Eosinophils and mast cells infiltrate the ESM bundles in pediatric patients with EoE, promote smooth muscle hyperplasia, and produce TGF- β 1, which causes transcription-dependent contraction of primary human ESM cells *in vitro*.^{10,25} To understand EoE pathogenesis and elucidate novel TGF- β 1 mechanisms, we used primary ESM and EoE myofibroblasts (EMFs) to demonstrate that the contraction-associated protein phospholamban (PLN) is a TGF- β 1 transcriptional target. PLN is required for proper cardiac muscle contraction, and human PLN mutations can be lethal.^{28,29} However, its role in ESM function in patients with EoE and its induction by TGF- β 1 have not been previously appreciated. For the first time, we show that TGF- β 1 induces and phosphorylates PLN in ESM cells and EMFs from patients with EoE. Patients with EoE had significantly higher PLN and phospho-PLN expression in the ESM *in vivo* compared with

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Abbreviations used

EMF:	Esophageal myofibroblast
EoE:	Eosinophilic esophagitis
ESM:	Esophageal smooth muscle
FACS:	Fluorescence-activated cell sorting
hpf:	High-power field
PLN:	Phospholamban
Serca2a:	Sarcoendoplasmic reticulum Ca^{2+} ATPase 2a
siRNA:	Small interfering RNA
α SMA:	α -Smooth muscle actin
TAK1:	TGF- β 1-activated kinase 1
TGF- β RI:	TGF- β receptor 1
TGF- β RII:	TGF- β receptor 2

that seen in healthy control subjects. PLN downregulation decreased TGF- β 1-mediated contraction. PLN induction required the canonical TGF- β 1 signaling pathway. As such, our findings reveal that PLN is a novel and previously unrecognized TGF- β 1 target that likely alters ESM function in the process of EoE-associated tissue remodeling.

METHODS**Cultured cells**

ESM cells. Primary human ESM cells were cultured according to the manufacturer's instructions (ScienCell, San Diego, Calif).

EoE fibroblasts. Fibroblasts were isolated from esophageal biopsy specimens from pediatric patients with EoE obtained during routine endoscopy with biopsy, dispersed with collagenase VIII (Sigma-Aldrich, St Louis, Mo), and cultured in smooth muscle cell media (ScienCell, Carlsbad Calif). Fibroblast phenotype was confirmed based on the production of collagen I, fibronectin, and appropriate morphology.

EMF cells. Esophageal myofibroblasts (EMFs) were defined as fibroblasts from patients with EoE producing α -smooth muscle actin (α SMA) after treatment with recombinant human TGF- β 1 (see below).

Treatment with TGF- β 1 and inhibitors

ESM cells or EMFs from patients with EoE were grown to 90% confluency and placed in serum-free medium overnight before treatment with 10 ng/mL recombinant human TGF- β 1. Inhibitors of TGF- β receptor 1 (TGF- β RI; SB431542; 10 μ mol/L; InvivoGen, San Diego Calif), TGF- β 1-activated kinase 1 (TAK1; 5Z-7-oxozeanol, 100 nmol/L; Sigma-Aldrich), or the appropriate vehicle control were added 3 hours before TGF- β 1. ESM cells were pretreated with TAK1 inhibitor, followed by treatment with recombinant IL-1 β for phosphorylated Jun kinase assays.

Cell contraction assays

Gel contraction assays with ESM or EMF were done, as previously described.¹⁰ Briefly, 1.25×10^5 cells were cultured in LPS-free collagen gels (Advanced BioMatrix, San Diego, Calif). Gels were treated with TGF- β 1, pharmacologic inhibitors, or both, and the area of the gels was quantified with a Chemidoc transilluminator and its accompanying software (Bio-Rad Laboratories, Hercules, Calif). Experiments were done in triplicate on at least 3 separate days. For gene silencing experiments, cells were transfected with an Amaxa Nucleofection system and small interfering RNA (siRNA) for PLN or a control nontargeting (NTG) siRNA (ThermoScientific Dharmacon, Lafayette, Colo), according to the manufacturer's guidelines. NTG is a control siRNA that does not target any gene as controlled for by using a genome-wide array.³⁰

Immunostaining and biopsy specimens from pediatric patients with EoE

EoE was defined as 15 or more eosinophils per high-power field (hpf) in an esophageal biopsy specimen on $\times 400$ light microscopy and hematoxylin-and-eosin stain in the presence of typical EoE symptoms and endoscopic features. Control was defined as no esophageal endoscopic abnormalities and eosinophil counts of 2 or more per hpf. Archived biopsy specimens were screened for the presence of smooth muscle by using our database of patients with EoE. Seven patients with EoE and 5 healthy control subjects with adequate muscularis mucosa for analysis were selected randomly from the University of California, San Diego/Rady Children's Hospital, San Diego, EoE database for PLN and phospho-PLN immunohistochemistry.

Five-micrometer sections of tissue were deparaffinized and hydrated before immunostaining, as previously described.² Immunocytochemistry on cells was performed with paraformaldehyde fixation and detergent treatment before primary and secondary antibodies. Esophageal biopsy specimens and primary cells were processed for immunohistochemistry or immunofluorescence by using primary antibodies (see Table E1 in this article's Online Repository at www.jacionline.org) specific for human PLN, phosphoserine-16-PLN, α SMA, TGF- β RI and TGF- β receptor 2 (TGF- β RII), sarcoendoplasmic reticulum Ca^{2+} ATPase 2a (Serca2a), or appropriate isotype control. The Vectastain ABC system (Vector Laboratories, Burlingame, Calif) was used for immunohistochemistry, as previously described.² Double and single immunofluorescence was performed, as previously described.¹⁰ Images were captured and analyzed by using image analysis (ImagePro; Media Cybernetics, Bethesda, Md), and slides were all quantified and analyzed under identical light or fluorescence microscopic conditions, including magnification, gain, camera position, and background illumination.

Quantitative PCR analysis

RNA was isolated by using chloroform extraction and isopropanol precipitation, converted to cDNA with Superscript II reverse transcriptase per the manufacturer's instructions (Invitrogen, Carlsbad, Calif), subjected to real-time quantitative PCR by using SYBR green (Qiagen, Hilden, Germany) and PLN-specific primers, and normalized to housekeeping genes (see Table E2 in this article's Online Repository at www.jacionline.org). Data are presented as $\Delta\Delta$ cycle threshold normalized to housekeeping genes. All dissociation curves had single peaks.

Immunoblots

Cells were harvested on ice by using RIPA lysis buffer (Teknova, Hollister, Calif) with phosphatase and protease inhibitors (Roche Applied Science, Indianapolis, Ind). Equivalent amounts of total protein were loaded into each well and electrophoresed on NuPAGE 4-12% Bis-Tris gels (Life Technologies, Grand Island, NY). Proteins were transferred to 0.2- μ m-pore nitrocellulose membranes (Life Technologies), blocked with 5% dry milk, incubated overnight with the primary antibody (see Table E1), and probed with the appropriate species-specific horseradish peroxidase-linked secondary antibody. Immunoblots were developed with Immuno-Star WesternC Kit (Bio-Rad Laboratories), imaged, and analyzed with the Chemidoc transilluminator and its accompanying software (Bio-Rad Laboratories).

Florescence-activated cell sorting analysis

After Fc block (Miltenyi Biotec, Auburn, Calif), 0.5 to 1×10^6 ESM cells or EMFs were stained for 30 minutes with TGF- β RI, TGF- β RII (see Table E1), and species-specific phycoerythrin- or allophycocyanin-conjugated secondary antibody. Cells were resuspended and washed in fluorescence-activated cell sorting (FACS) buffer (2% FBS and 1 mol/L sodium azide in $1 \times$ dPBS) and used in standard indirect flow cytometry experiments. For TGF- β RII surface staining, cells were incubated with Fc receptor blocking antibody before primary and secondary antibodies

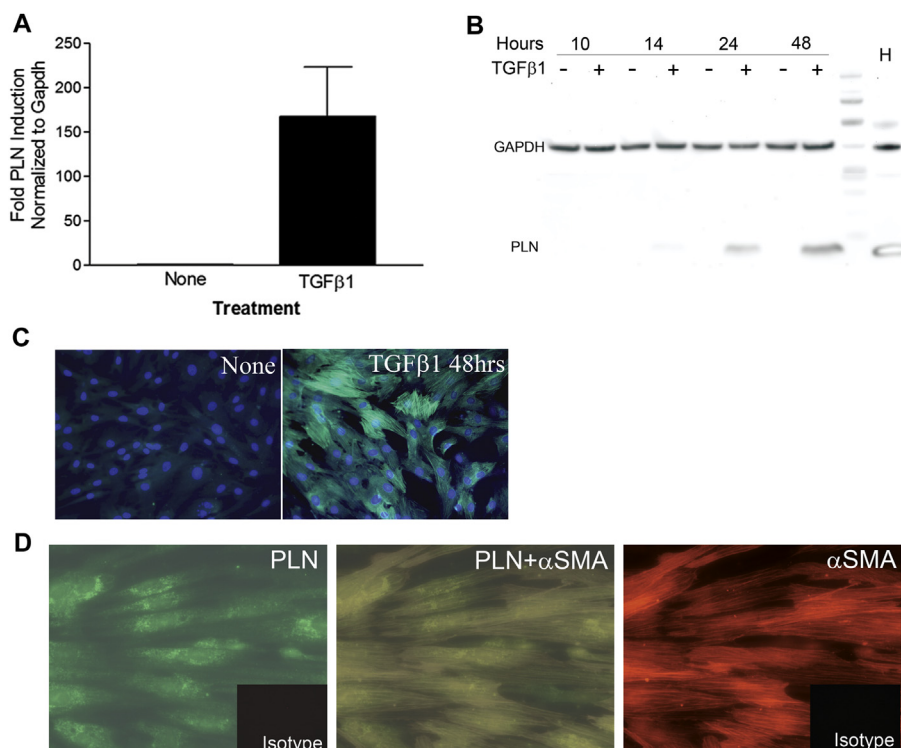


FIG 1. TGF- β 1 induces PLN in ESM cells and EMFs from patients with EoE. **A** and **B**, PLN mRNA (Fig 1, **A**) and protein (Fig 1, **B**) are detected in primary ESM cells after TGF- β 1 treatment. **H**, Cardiac extract positive control. **C** and **D**, Primary fibroblasts from patients with EoE express α SMA (green; blue, nuclear 4'-6-diamidino-2-phenylindol dihydrochloride; Fig 1, **C**) and PLN (green), which colocalizes (yellow) with α SMA (red) on TGF- β 1 treatment (Fig 1, **D**). Protein molecular weight ladder shows PLN monomer of less than the 5-kDa marker. Isotype control (insets) shows no staining. *Gapdh*, Glyceraldehyde-3-phosphate dehydrogenase.

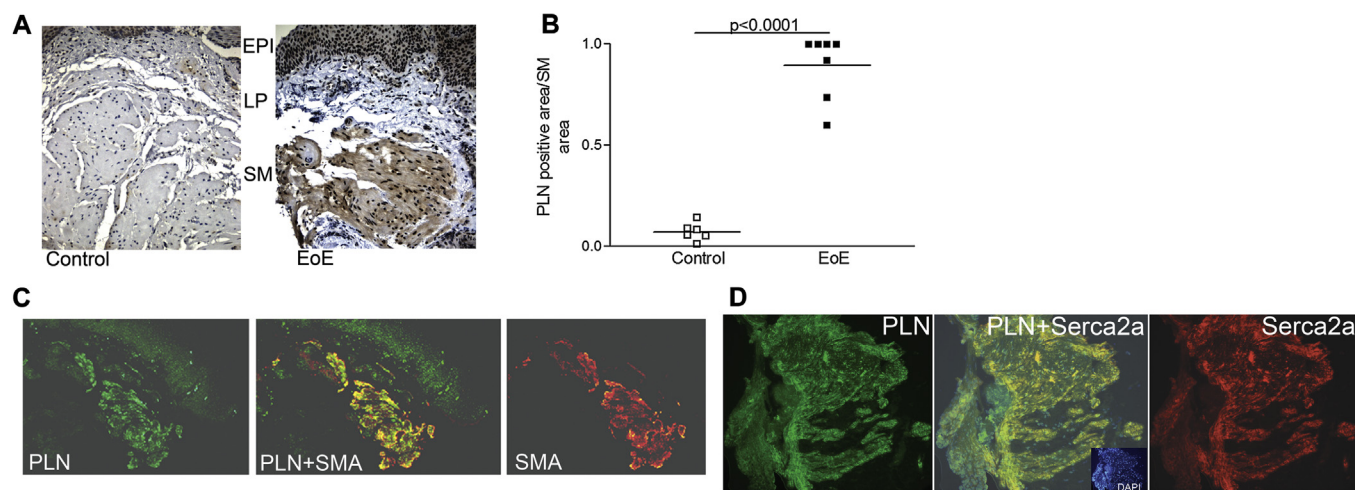


FIG 2. PLN is expressed in smooth muscle from patients with EoE and localizes with Serca2a. **A** and **B**, Representative image (Fig 2, **A**) and quantification (Fig 2, **B**) of smooth muscle PLN in control biopsy specimens and esophageal biopsy specimens from patients with EoE. **C**, PLN (green) colocalizes (yellow) with α SMA (red). **D**, PLN (green) colocalizes (yellow) with Serca2a (red) in pediatric patients with EoE. *Inset* shows nuclear 4'-6-diamidino-2-phenylindol dihydrochloride staining. *EPI*, Epithelium; *LP*, lamina propria; *SM*, smooth muscle.

(allophycocyanin-conjugated IgG; BioLegend, San Diego, Calif). For TGF- β RI intracellular staining, cells were treated with blocking antibody and then incubated with TGF- β RI and species-specific secondary antibodies (phycoerythrin conjugated; eBioscience, San Diego, Calif).

Mouse IgG₁ (eBioscience) and rabbit IgG (GeneTex, Irvine, Calif) were used as isotype control antibodies. The samples were analyzed with an AccuriC6 flow cytometer. Further analyses were performed with FlowJo software (Tree Star, Ashland, Ore).

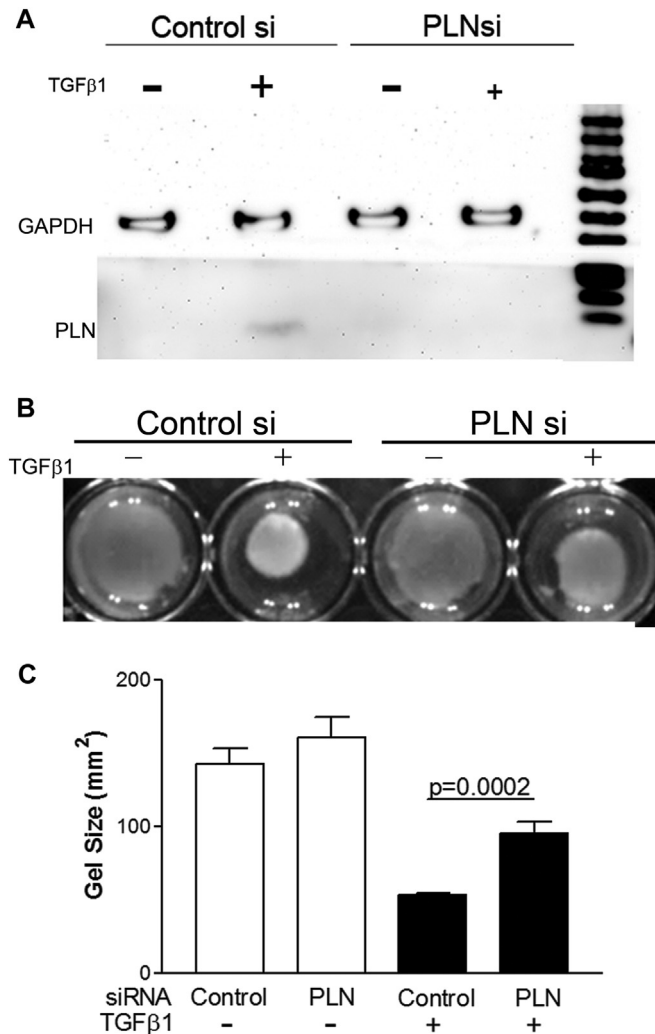


FIG 3. PLN downregulation significantly decreases esophageal EMF cell contraction in patients with EoE. **A**, PLN siRNA inhibits TGF- β 1-induced PLN expression (representative Western blot). *GAPDH*, Glyceraldehyde-3-phosphate dehydrogenase. **B** and **C**, TGF- β 1-induced contraction of EMFs from patients with EoE (representative contraction gel; Fig 3, **B**) is significantly decreased by PLN siRNA but not by control siRNA (Fig 3, **C**). Graphs represent means and SEs.

Statistics

All statistical analyses and graphing were carried out with GraphPad Prism software (GraphPad Software, San Diego, Calif). Comparisons between 2 groups were done with the Student *t* test for unpaired variables. A 2-tailed *P* value of less than .05 was considered statistically significant.

Human subjects

All aspects of the study received institutional review board approval by the University of California, San Diego (University of California, San Diego, protocol no. 091485).

RESULTS

TGF- β 1 induces PLN in human ESM cells

Our prior studies demonstrated that primary human ESM cells contract in a transcription-dependent manner when cultured in the presence of TGF- β 1.¹⁰ However, potential contraction-associated targets were not clear. We found that TGF- β 1 treatment of ESM

cells induced PLN mRNA 167-fold in human ESM cells (Fig 1, **A**). PLN is a pentameric sarcoplasmic reticulum protein that regulates the function of Serca2a and is essential for proper cardiac contractility.³¹ As such, it was a potential candidate protein involved in TGF- β 1-induced ESM contraction. PLN mRNA was undetectable at baseline and became increasingly abundant beginning 4 hours after and continuing through 48 hours of TGF- β 1 treatment (data not shown). Similarly, induction of PLN mRNA was seen in EMFs from patients with EoE after TGF- β 1 treatment (see Fig E1 in this article's Online Repository at www.jacionline.org).

PLN protein was undetectable by using immunoblotting at baseline in ESM cells but became detectable after 14 hours of TGF- β 1 and continued to be expressed through 48 hours of treatment (Fig 1, **B**). Because primary ESM cells from pediatric patients with EoE are difficult to culture directly from biopsy specimens, we used primary esophageal fibroblasts from patients with EoE that transform to EMFs in the presence of TGF- β 1. EMF phenotype was verified by increased α SMA expression after 24 or more hours of TGF- β 1 treatment (Fig 1, **C**). EMFs from patients with EoE demonstrated increased PLN protein levels, as well as coexpression of PLN and α SMA, after TGF- β 1 treatment, demonstrating that conversion of fibroblasts from patients with EoE to an EMF phenotype induces a protein cascade that includes PLN induction (Fig 1, **D**, and see Fig E1). Taken together, these data suggest a role for PLN in ESM cell and EMF function.

Biopsy specimens from pediatric patients with EoE express PLN in the muscularis mucosa

Because TGF- β 1-expressing mast cells and eosinophils infiltrate the muscularis mucosa in patients with EoE¹⁰ and because TGF- β 1 induced PLN expression, we hypothesized that patients with EoE would express increased PLN levels in their ESM bundles as opposed to control subjects. EoE was defined as 15 or more eosinophils per hpf (mean, 76 eosinophils/hpf). The majority of subjects (86%) had typical endoscopic findings (furrows, plaques, and/or pallor) and were allergic (71%). The mean age of the subjects whose biopsy specimens were used was 4.8 years. Eighty-six percent had vomiting, 29% had regurgitation, and 14% had gagging. Although dysphagia can be difficult to discern in a young population, these symptoms could be clinical manifestations of dysmotility. In addition, 14% complained of dysphagia, and 43% had abdominal pain and/or anorexia/early satiety with poor weight gain.

Immunohistochemistry and image analysis determined that patients with EoE had significantly more PLN in the muscularis mucosa compared with control subjects (Fig 2, **A** and **B**). PLN and α SMA were coexpressed in biopsy specimens from pediatric patients with EoE, confirming that the ESM bundles were the cellular location of PLN expression in patients with EoE (Fig 2, **C**). These findings are consistent with the concept that TGF- β 1 produced by smooth muscle-infiltrating inflammatory cells in patients with EoE can induce PLN expression and that the presence of PLN reflects a pathologic state in patients with EoE.

PLN regulates contraction by altering sarcoplasmic reticulum calcium stores because of Serca2a inhibition.³¹ Esophageal biopsy specimens from patients with EoE showed colocalization of PLN and Serca2a in ESM cells (Fig 2, **D**), supporting the hypothesis that PLN could regulate Serca2a function and calcium flux in an EoE inflammatory state.

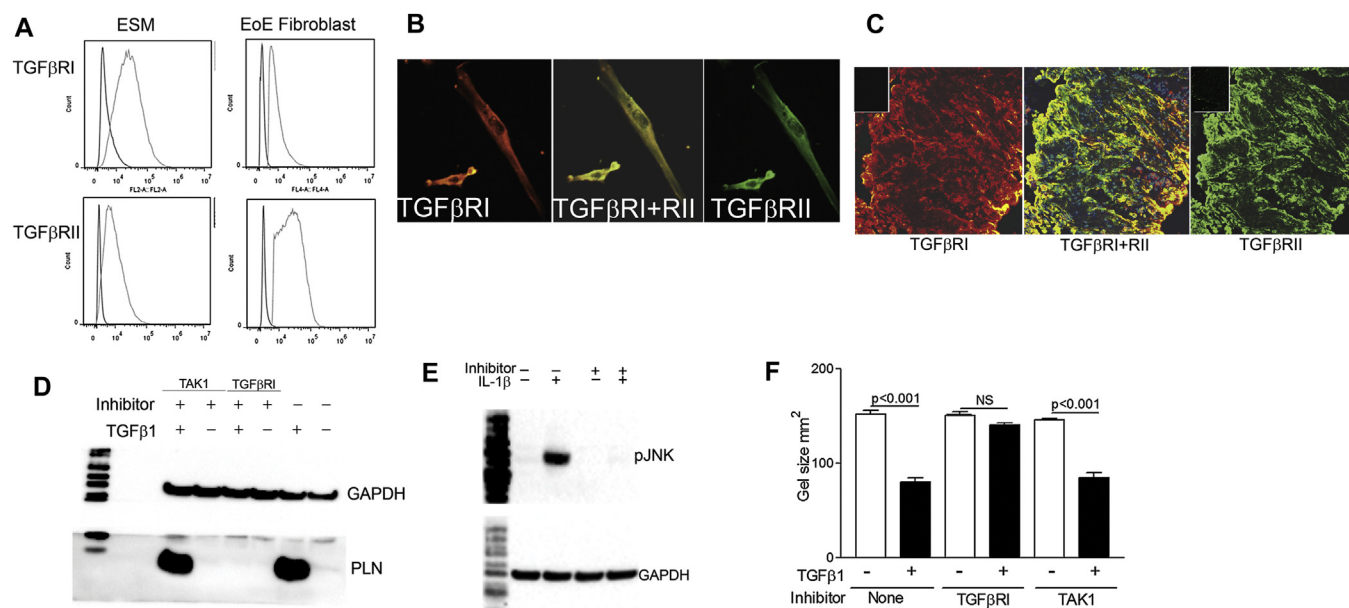


FIG 4. TGF- β RI and TGF- β RII are expressed on ESM cells and required for TGF- β 1-mediated contraction, as well as PLN phosphorylation. **A**, FACS analysis (black plot, isotype control; gray plot, specific antibody) demonstrates that ESM cells and fibroblasts from patients with EoE express TGF- β RI and TGF- β RII. **B**, TGF- β RI (red) and TGF- β RII (green) are coexpressed (yellow) on fibroblasts from patients with EoE. **C**, Representative image of EoE biopsy specimen shows colocalization (yellow) of TGF- β RI (red) and TGF- β RII (green) receptors in ESM cells (insets show isotype control). **D** and **E**, TGF- β RI inhibitor, but not TAK1 inhibitor, abolishes PLN expression induced by TGF- β 1 (Fig 4, **D**), but TAK1 inhibitor does block c-Jun N-terminal kinase phosphorylation by IL-1 β (Fig 4, **E**). GAPDH, Glyceraldehyde-3-phosphate dehydrogenase. **F**, TGF- β RI inhibitors block ESM contraction, but TAK1 inhibitor does not.

PLN is required for TGF- β 1-induced contraction

On the basis of the known function of PLN, its expression in the pathogenic EoE state, and its induction by TGF- β 1, we hypothesized that TGF- β 1's effects on contraction would depend on PLN. We transfected TGF- β 1-treated EMFs from patients with EoE with an NTG control siRNA³⁰ or PLN-specific siRNA to block the production of PLN protein in EMFs from patients with EoE (Fig 3, **A**). Collagen gels impregnated with PLN-specific siRNA-treated EMFs had significantly less TGF- β 1-induced contraction compared with EMFs that were transfected with control siRNA (Fig 3, **B** and **C**). Thus PLN could be a previously unrecognized mechanism for TGF- β 1-induced ESM contraction in patients with EoE.

TGF- β RI signals are required for PLN induction

To understand whether PLN expression depended on the canonical TGF- β 1 signaling pathway, we used pathway-specific pharmacologic inhibitors. FACS analysis demonstrated that TGF- β RI and TGF- β RII are expressed on both fibroblasts and ESM cells from patients with EoE (Fig 4, **A**), and double immunofluorescence with TGF- β RI- and TGF- β RII-specific antibodies showed colocalization on smooth muscle from patients with EoE *in vivo* and on EMFs from patients with EoE, verifying that both receptors were expressed together to allow for proper TGF- β 1 signals (Fig 4, **B** and **C**). The TGF- β RI-specific inhibitor SB431542 caused complete abolishment of PLN expression induced by TGF- β 1 (Fig 4, **D**). Blockade of TGF- β RI signaling also completely abrogated TGF- β 1-induced contraction of both ESM cell- and EMF-impregnated collagen gels (Fig 4, **F**, and see Fig E2 in this article's Online Repository at

www.jacionline.org). By contrast, pharmacologic inhibition of the noncanonical TAK1 pathway did not decrease PLN expression (Fig 4, **D**), although it did inhibit c-Jun N-terminal kinase phosphorylation (Fig 4, **E**), providing proof that noncanonical TGF- β 1 pathways are functional in ESM cells. As would be predicted by an inability to block TGF- β 1 induction of PLN, TAK1 inhibition did not alter TGF- β 1-mediated ESM or EMF contraction (Fig 4, **F**, and see Fig E2).

Serine phosphorylation alters the function of PLN. Therefore we evaluated the phosphorylation state of PLN in ESM cells from patients with EoE. Phospho-serine-PLN- and phospho-threonine-PLN-specific antibodies and extracts from TGF- β 1-treated and untreated EMFs and ESM cells (cardiac lysates were used as a positive control) demonstrated that phospho-serine-16 PLN was detectable after TGF- β 1 treatment but phospho-threonine-17-PLN was not (Fig 5, **A** and **B**). Phospho-serine-16-PLN was also detected in the muscularis mucosa of patients with EoE but not in nondiseased control subjects, thereby verifying the presence of phosphorylated PLN in the EoE disease state (Fig 5, **C**).

DISCUSSION

In this study we demonstrate a number of novel findings. This study is the first to document a potential mechanism for TGF- β 1-induced ESM contraction in patients with EoE. PLN induction and phosphorylation by TGF- β 1 have not been previously documented. In addition, we show that TGF- β 1 signaling is necessary to induce PLN in ESM and EMF cell cultures. It is possible that TGF- β 1 could alter both smooth muscle contraction and general tissue contraction during a

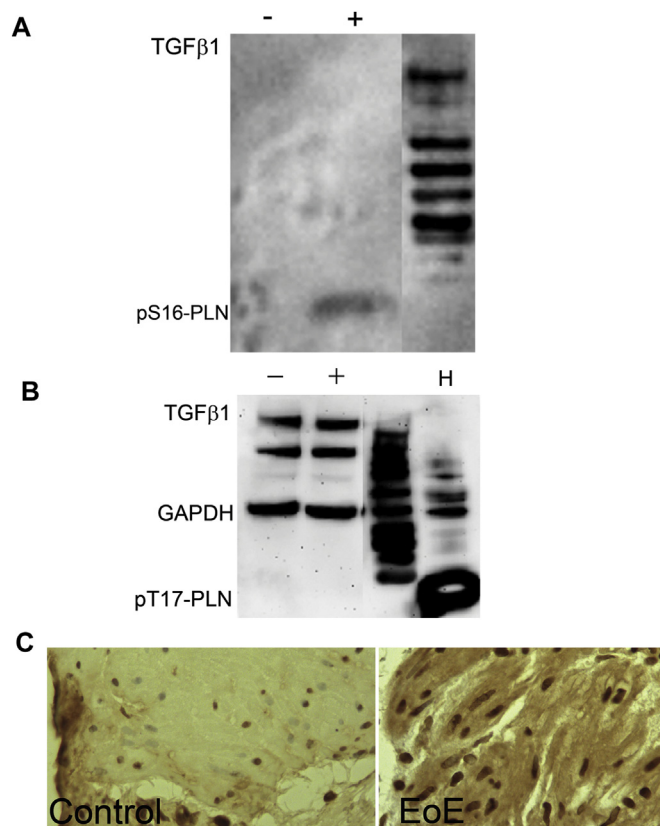


FIG 5. TGF- β 1 causes PLN phosphorylation. **A** and **B**, Phospho-serine-16 is detectable after TGF- β 1 treatment (Fig 5, **A**), but phospho-threonine-17 is not (Fig 5, **B**). The heart (**H**) was used as a positive control to detect phospho-threonine-17-PLN. *GAPDH*, Glyceraldehyde-3-phosphate dehydrogenase. **C**, A representative image demonstrates that phospho-serine-16 is expressed in ESM from patients with EoE but not in control ESM.

process akin to wound healing through its effects on ESM cells and EMFs (Fig 6). Because the human PLN gene contains 2 well-conserved Smad consensus binding sequences, PLN is a feasible direct transcriptional TGF- β 1 target and might provide a new therapeutic target in patients with EoE. Because TGF- β 1 also uses noncanonical signaling pathways in other systems, such as JunB for uterine smooth muscle contraction,³² it will be interesting to understand the pSmad-dependent and independent mechanisms for TGF- β 1-mediated contraction and PLN induction.

Understanding the mechanisms underlying smooth muscle changes in patients with EoE is important because both dysphagia- and nonstricture-associated food impactions likely occur as a result of alterations in smooth muscle.^{6,13,14,18,19,33-35} Although esophageal fibrosis with resultant fixed esophageal strictures/narrowing contributes to dysphagia,^{3,4,8,9} a body of work supports a role for altered motor function in patients with EoE.¹⁴ Structural analyses demonstrate that eosinophils and mast cells are present in the deep muscular layers,³⁶⁻³⁸ and murine models demonstrate the effects of mast cells on smooth muscle proliferation.¹¹ Indeed, animal studies have shown that the mechanisms that promote motor abnormalities can be distinct from those that drive esophageal strictures.²⁴ In pediatric patients with EoE, 24-hour monitoring of esophageal motility demonstrated that transient motility disturbances reflected clinical dysphagia.¹⁸ Studies using stationary manometry, which

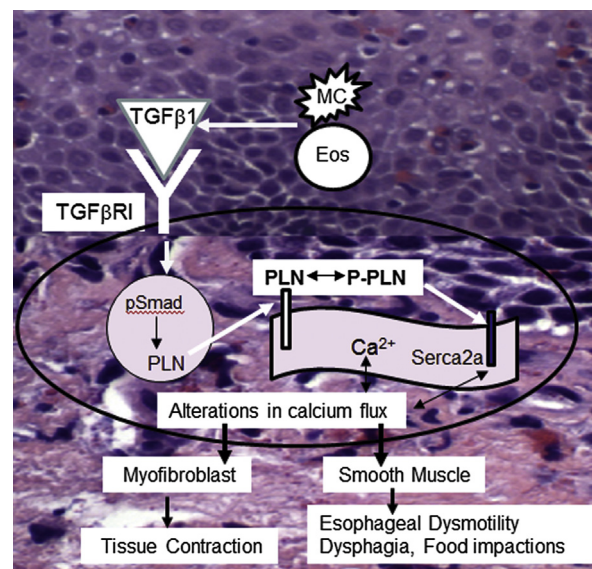


FIG 6. Model of the pathway by which TGF- β 1 could regulate or alter ESM and myofibroblast function. TGF- β 1 produced by mast cells and eosinophils binds to its receptor (TGF- β RI), causing PLN transcription through the phosphorylated Smad pathway and colocalization of PLN with the calcium channel Serca2a. PLN phosphorylation (P-PLN) and dephosphorylation change calcium flux into the sarcoplasmic reticulum through Serca2a. Intracellular calcium changes could cause ESM cell and myofibroblast contraction to cause esophageal dysmotility and tissue contraction. MC, Mast cell.

is limited to an isolated assessment of circular muscle function, have determined variable patterns of esophageal dysmotility in subsets of patients with EoE, including sustained, irregular, and/or ineffective contractions, achalasia, and nutcracker esophagus.^{13,16,19,20} In contrast, studies that assess both the concentric and longitudinal esophageal layers demonstrated uniform, ineffective longitudinal muscle function and muscle layer asynchrony, with no changes in concentric smooth muscle function.¹⁵ As such, there are clearly patients with EoE who have dysmotility. Thus the definition of a dysmotile EoE phenotype will be critical because it might indicate the opportunity to target novel molecules, such as PLN.

Increased TGF- β 1 levels are likely to induce a cascade of genes that can regulate ESM cell and EMF function. The basal expression of ESM TGF- β 1 receptors coupled with the influx of TGF- β 1-producing mast cells and eosinophils into the smooth muscle of patients with EoE provides a cellular mechanism explaining the physiologic and pathogenic consequences of TGF- β 1.^{10,11,36} Because smooth muscle inflammation occurs in patients with EoE, PLN induction would not occur in normal noninflamed smooth muscle. Indeed, PLN expression is readily detected in the muscularis mucosa of patients with EoE and is very low in control subjects. Because PLN blockade significantly decreases TGF- β 1-induced contraction, we provide evidence that PLN is likely a necessary part of TGF- β 1's effects on smooth muscle. However, because PLN blockade partially reduces contraction, it appears to be an integral, but not the only, component for the effects of TGF- β 1. Indeed, deciphering PLN-dependent and independent pathways on EoE smooth muscle function will be of interest to better understand disease pathogenesis.

PLN is most completely characterized in the heart, where it is a component of the Serca2a-PLN calcium regulatome.³¹ Both null and missense mutations in the human PLN gene cause cardiomyopathy, heart failure, cardiomyocyte enlargement, decreased contractile function, and electrophysiologic remodeling.^{28,39,40} PLN has been described in the murine gastric smooth muscle, as well as in the airways of β receptor–overexpressing mice. The expression patterns and effects of PLN on smooth muscle function might vary by disease state and inflammatory milieu. For example, nonasthmatic human airway smooth muscle cells express PLN mRNA but not PLN protein, whereas isolated airway smooth muscle cells have a reduction in Serca2a levels after treatment with IL-13 and TNF- α . In addition, transgenic mice overexpressing the β_2 -adrenergic receptor have decreased levels of PLN, and PLN-deficient mice have reduced airway smooth muscle contractile responses to methacholine.^{41,42} Given our current study on EoE, it would be of interest to know whether PLN levels are altered in human asthmatic airways and whether TGF- β 1 can regulate PLN levels in airway smooth muscle cells.

Prior studies also support a role for PLN in gastrointestinal motility. In murine gastric smooth muscle phosphorylation of PLN by CamKinase II is associated with relaxation, and PLN-deficient mice showed increased slow waves, demonstrating that PLN is involved in the electrical activity of gastric smooth muscle.^{43,44} In the neonatal guinea pig gallbladder decreased PLN levels are associated with decreased contraction compared with the adult gallbladder, and cholecystokinin (CCK) causes both PLN phosphorylation and increased contraction.⁴⁵ In a dextran sodium sulfate (DSS)-induced colitis model decreased colonic contractility was associated with decreased PLN levels.⁴⁶ Although a study of subjects with achalasia demonstrated an inability to detect PLN by using immunoblotting in human biopsy tissues, it did demonstrate decreased levels of the calcium-handling proteins calreticulin and calsequestrin in subjects with achalasia.⁴⁷ Given that eosinophil products can alter esophageal contractility,³⁸ it is possible that subjects with esophageal eosinophilia and achalasia could also have alterations in PLN levels. Although animal models might not entirely align with human PLN function (eg, human subjects with PLN deficiency have heart failure, whereas mice do not exhibit a lethal phenotype⁴⁰), it is clear that PLN and its alterations are important in smooth muscle calcium handling, electrical activity, and contraction.

TGF- β 1-induced PLN phosphorylation on serine-16 was dependent on TGF- β RI but not on the noncanonical TAK1 pathway. This is consistent with the serine phosphorylation signals that occur through TGF- β RI receptor. Both the levels and phosphorylation state of PLN affect its inhibitory effects on Serca2a.³¹ Baseline Serca2a expression coupled with the onset of PLN expression in patients with EoE could provide a significant functional insult on calcium homeostasis in patients with EoE. It is also important to consider that by altering calcium flux, which regulates a number of physiologic processes, PLN could play a more global role in the EoE structural remodeling process in smooth muscle. In addition, because TGF- β 1 constitutes a pathway to fibrosis and remodeling in patients with asthma and heart and renal failure, our findings could have important implications for other disease states. Indeed, current clinical trials in patients with abnormal PLN expression are aimed at reducing not only cardiac dysfunction but also cardiac fibrosis.

In conclusion, our data propose a new paradigm for esophageal remodeling in which inflammatory cell–derived TGF- β 1 acts on

the ESM to induce and phosphorylate PLN and alter calcium flux, as regulated through Serca2a. The fact that the effects of TGF- β 1 on PLN induction and phosphorylation occur slowly is consistent with the chronic disordered contraction and smooth muscle remodeling seen in patients with EoE. The relationship of this mechanism to other diseases, as well as to other structural remodeling features in patients with EoE, warrants further investigation and could provide novel and currently unappreciated therapeutic options for patients with chronic allergic esophagitis.

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Clinical implications: EoE is accompanied by smooth muscle remodeling and functional alterations, but the mechanisms are not known. We show that PLN is regulated by TGF- β 1, and as such, it might provide a novel therapeutic target to improve esophageal dysmotility and clinical dysphagia.

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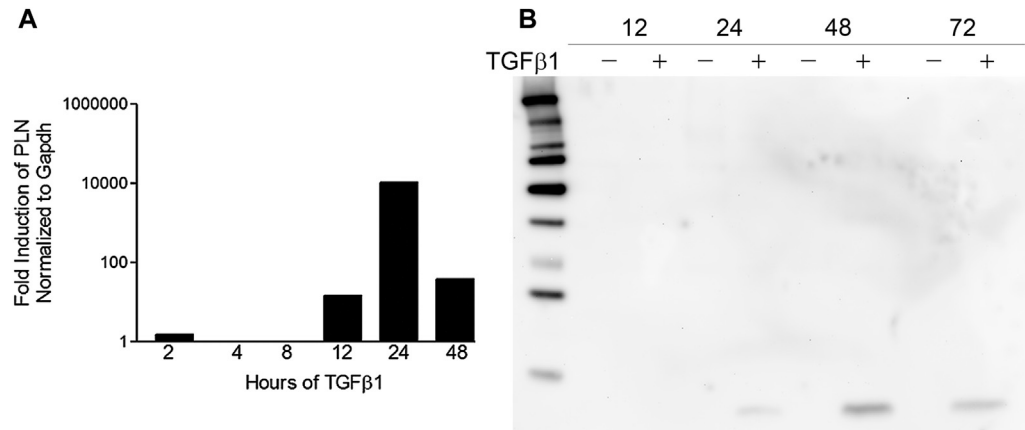


FIG E1. TGF- β 1 induces PLN in EMFs from patients with EoE. Representative time course of PLN mRNA (**A**) and protein (**B**) after treatment of EMFs from patients with EoE is shown. *Gapdh*, Glyceraldehyde-3-phosphate dehydrogenase.

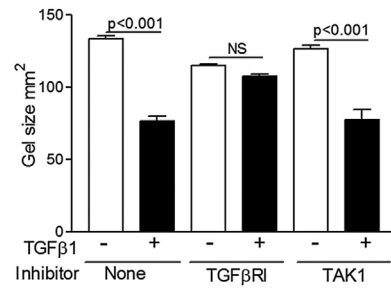


FIG E2. TGF- β RI but not TAK1 inhibition abolishes EMF cell contraction in patients with EoE. EMF cells from patients with EoE treated with TGF- β 1 have EMF-impregnated gel contraction that is inhibited by pharmacologic inhibition of TGF- β RI but not TAK1. *NS*, Not significant.

TABLE E1. Antibodies used

Antibody	Catalog no., concentration (manufacturer)	Application
PLN	HPA026900 1:250 (Sigma-Aldrich, St Louis, Mo)	IHC/IF
Phospho-serine-16 PLN	Ab15000 1:200 (Abcam, Cambridge, Mass)	IHC
α SMA	A2547, 1:500 (Sigma-Aldrich)	IHC/IF
Serca2a	Ab2861, 1:1000 (Abcam)	IF
TGF- β RI	Ab31013 1:50 (Abcam)	IHC/IF
TGF- β RII	Ab78419, 1:100 (Abcam)	IHC/IF
PLN	Ab2865, 1:750 (Abcam)	Immunoblot
Phospho-serine-16-PLN	NBP40742, 1:1000 (Novus Biologicals, Littleton, Colo)	Immunoblot
Phospho-threonine-17-PLN	SC170424R, 1:500 (Santa Cruz Biotechnology, Dallas, Tex)	Immunoblot
JNK	56G8, 1:2000 (Cell Signaling, Danvers, Mass)	Immunoblot
Phospho-JNK	81E11, 1:2000 (Cell Signaling)	Immunoblot
GAPDH	SC-32233, 1:2000 (Santa Cruz Biotechnology)	Immunoblot
TGF- β RI	ab31013m (Abcam)	FACS
TGF- β RII	ab78419 (Abcam)	FACS

GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; *IF*, immunofluorescence; *IHC*, immunohistochemistry; *JNK*, c-Jun N-terminal kinase.

TABLE E2. Primer sequences used for quantitative PCR

Primer	Forward	Reverse
PLN	AAACTCCCCAGCTAAACACC	GAACTTCAGAGAAGCATCACGATGATA
RPL13A	CATAGGAAGCTGGGAGCAAG	GCCCTCCAATCAGTCTTCTG
GAPDH	TGGTATCGTGGAAG-GGACTCAT	ATGCCAGTGAGCTTCCCGTTC

GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.