

Letter to the Editor

Rigid substrate induces esophageal smooth muscle hypertrophy and eosinophilic esophagitis fibrotic gene expression

To the Editor:

Eosinophilic esophagitis (EoE) is a chronic, food antigen-mediated disease associated with esophageal fibrosis and dysmotility.¹ Esophageal studies using ultrasounds and endoscopic functional luminal probes show increased esophageal rigidity and thickening of the smooth muscle (SM) layers, indicating hypertrophy.²⁻⁴ During EoE progression, the esophagus undergoes substantial tissue remodeling with fibrosis, leading to clinical complications including strictures and food impactions.^{1,4} EoE is induced by inflammation, but the consequences of tissue remodeling can remain even after the inflammation is treated, suggesting the role of a fibrotic extracellular matrix (ECM) in the chronic nature of the disease. Fibrosis promotes remodeling of ECM, a substrate for adherent cells composing the tissue, often resulting in increased ECM rigidity. Changes in substrate rigidity have been shown to affect multiple cellular functions important for development, tissue homeostasis, and progression of some diseases including cancer.⁵⁻⁷ Given the increased esophageal rigidity in EoE, we investigated the effect of cell substrate rigidity on the morphology, size, and gene transcription of cultured normal longitudinal smooth muscle (LSM) cells.

To control the rigidity of the substrate for primary human donor esophagus-derived LSM cells, we used SoftSubstrates silicone gels (MuWells, San Diego, Calif) of defined, physiologically

relevant rigidities (Young's Moduli, E , of 0.8, 3, 24, and 48 kPa) (see this article's [Methods](#) section in the Online Repository at www.jacionline.org). We found that esophageal LSM cells cultured on a stiffer substrate exhibited decreased cell elongation, more pronounced actin filaments, and larger size compared with cells plated on softer substrates (Fig 1, A and B). The cell-spreading area was also significantly greater on increasingly rigid matrix ($P = .003$) (Fig 1, C). Taken together, our data indicate that SM cells on a rigid matrix have a phenotype consistent with increased contraction and/or hypertrophy.

Phospholamban (PLN), an integral membrane protein regulating the sarcoendoplasmic reticulum calcium channel Serca2, is essential for cardiac contractility and is upregulated in the esophageal muscularis mucosa in pediatric subjects with EoE.^{8,9} Our recent work has also identified PLN as an important player in TGF β 1-mediated esophageal SM cell and myofibroblast contraction.⁸ To investigate the effects of a rigid matrix on PLN expression, we measured the transcriptional level of PLN in LSM cells plated on substrates of $E = 0.8, 3, 24$, and 48 kPa and found it to be monotonically increased by substrate rigidity (Fig 2, A). To understand the role of PLN in regulating SM cell size and morphology, we used a stable transgenic primary human esophageal SM cell line expressing PLN under the control of a doxycycline-inducible promoter (see this article's [Methods](#) section in the Online Repository). Using these cells, we found that SM cells overexpressing PLN had decreased elongation and increased cell size on flow cytometry analysis (Fig 2, B and C). Therefore, upregulation of PLN expression is sufficient to induce morphological changes in SM similar to those observed on rigid substrates.

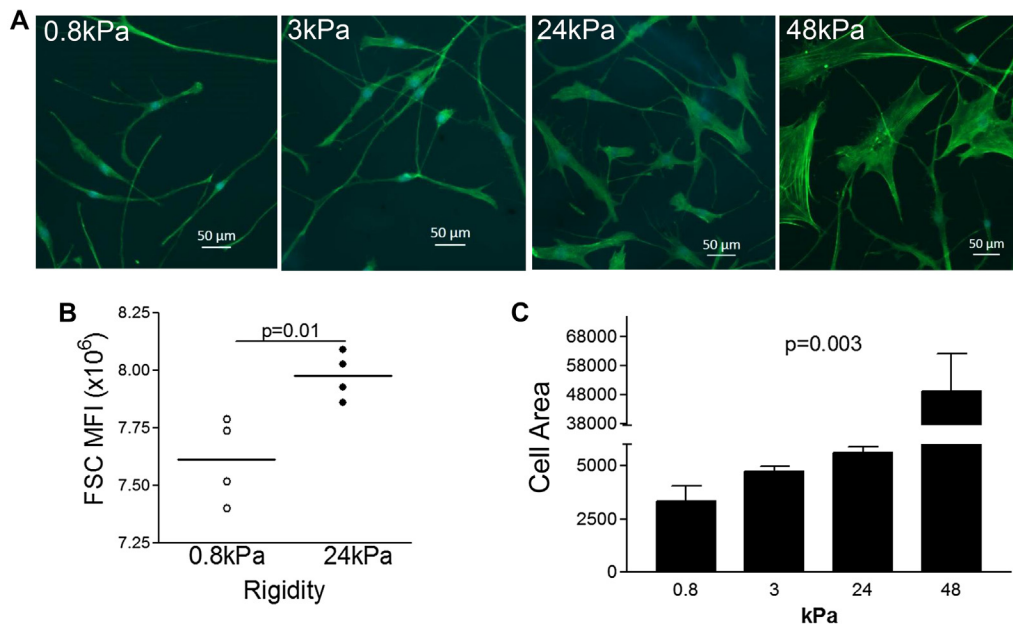


FIG 1. Morphology of LSM cells on substrates of different rigidity. Representative image of phalloidin stain (green) of cells cultured on silicone gels of indicated elastic moduli (0.8, 3, 24, and 48 kPa) (A). FACS analysis for cell size (relative units) was done using forward scatter and mean fluorescence intensity (MFI) on live cells using Flowjo software (Tree Star, Ashland, Ore) using cells cultured on 0.8- and 24-kPa substrates. Each dot represents a biological replicate. Lines represent the mean. The P value was calculated using an unpaired t test (B). Quantification of cell area by the ratio of the area of phalloidin staining and cell numbers (quantified by blue nuclei) on substrates of indicated rigidities (0.8-48 kPa). The P value was calculated using the ANOVA test (C). FACS, Fluorescence-activated cell sorting; FSC, forward scatter.

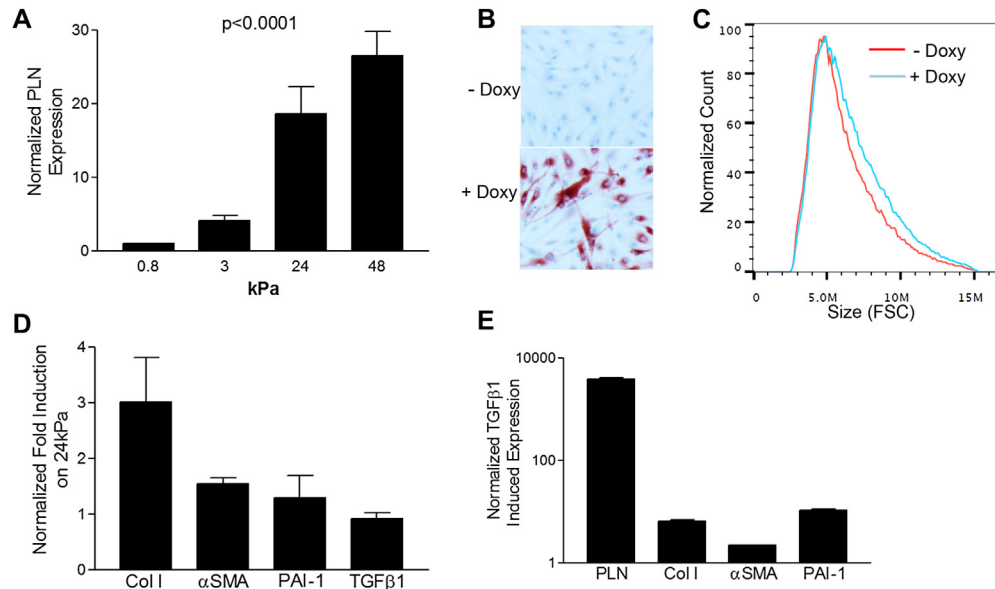


FIG 2. Gene transcription and cell size in response to rigid substrate, PLN, or TGFβ1. PLN induction in LSM cells cultured on substrates of indicated rigidities (0.8–48 kPa). The *P* value was calculated using the ANOVA test (A). Doxycycline (Doxy) treatment of PLN-inducible transgenic esophageal SM cells shows upregulation of PLN expression (red stain) (B) and increased cell size using forward scatter (C). Normal LSM cells have increased gene transcription of indicated genes expressed as a ratio of gene induction on hard (*E* = 24 kPa) versus soft (*E* = 0.8 kPa) substrates (D). Fold increase in LSM gene expression induced by TGFβ1 treatment (E). mRNA levels for all conditions were normalized to the housekeeping gene RPL13A. FSC, Forward scatter.

The pattern of gene expression in LSM cultured on rigid cell substrate was strikingly reminiscent of a subset of TGFβ1-induced genes including PLN, collagen I, and αSMA (Fig 2, D and E). However, the expression of TGFβ1 itself and other TGFβ1-induced genes such as plasminogen activator inhibitor-1 was not affected by the rigidity of the cell substrate (Fig 2, D). Therefore, cellular responses to substrate rigidity are likely to be controlled by signaling pathways with upstream molecular player(s) other than TGFβ1.

In summary, we report here that a rigid cell substrate induces morphological and transcriptional changes in SM cells of the esophagus consistent with EoE. This finding challenges the paradigm of EoE pathogenesis as almost exclusively inflammation-dependent. In addition, the induction of collagen I, a structural component of the esophageal ECM, by a rigid matrix suggests that rigidity can potentially cause a positive feedback loop for fibrosis. We also demonstrate that PLN may be a key molecular player in rigid substrate-induced cellular hypertrophy because its expression increased monotonically with increasingly rigid substrate in parallel with cell size. Moreover, we demonstrated that PLN overexpression is sufficient to promote SM cell hypertrophy. These results suggest that successful EoE treatment might require therapies that reverse the fibrotic changes in the ECM to minimize the effects of the rigid matrix on SM and that PLN may be a potential molecular target for these therapies. A focus on both inflammation-independent and inflammation-dependent esophageal rigidity may be needed to limit the onset, halt the progression, or reverse the path to a fibrostenotic esophagus in EoE.

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METHODS

To derive primary esophageal LSM cells, LSM bundles were isolated from human donor esophagi (under University of California at San Diego Institutional Review Board exempt protocol 130835) followed by collagenase D digestion (Roche, Mannheim, Germany). SoftSubstrates were coated with 1.6 $\mu\text{g/mL}$ solution of collagen I (Advanced Biomatrix, San Diego, Calif) in pH 7.4 PBS for 60 minutes at 37°C followed by plating and culturing of cells. For morphological analyses, cells were cultured for 24 to 48 hours followed by fixation and staining for actin filaments with phalloidin-488 (Life Technologies, Grand Island, NY) and nuclei with 4'-6-diamidino-2-phenylindole, dihydrochloride (Vector, Burlingame, Calif) using the manufacturers' protocols. Fluorescence micrographs were processed and analyzed using ImageJ (National Institutes of Health). FACS analysis was performed to assess cell size on 0.8- and 24-kPa matrixes as previously described.^{E1} For transcriptional analyses, cells were harvested after 96 hours of culture followed by reverse transcription quantitative PCR analyses. Gene expression studies were repeated in technical duplicates to triplicates and in biologic triplicates.

To create stable cell lines expressing PLN, full-length PLN cDNA was cloned downstream of the Tet Responsive Element of the HIV1 vector

backbone plasmid, pLVX-Tight-Puro (Clontech, Mountain View, Calif), which also expresses a puromycin-resistance gene driven by PGK promoter. Full-length PLN coding region was cloned by PCR using a human PLN cDNA containing plasmid as the template (OriGene catalog no. RC202712). Esophageal SM cells were coinfecting with LV-Tet-PLN and LV-Tet-On viruses at a multiplicity of infection of 10 each in the presence of polybrene (4 $\mu\text{g/mL}$). At 24 hours after lentivirus infection, cells were fed with fresh medium containing puromycin (1 $\mu\text{g/mL}$) and G418 (200 $\mu\text{g/mL}$) and resistant cells were continued in culture in low selection media using G418 and puromycin and passaged in SM cell media (ScienCell, San Diego, Calif) with serum and growth factors. Cells were treated with 2 $\mu\text{g/mL}$ doxycycline for 72 hours before fluorescence-activated cell sorting analysis and PLN expression was verified using quantitative PCR, immunoblotting, and immunohistochemistry (Fig 2, B).

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