Supporting Information

Porcine Vocal Fold Lamina Propria-Derived Biomaterials Modulates TGF-β1 Mediated Fibroblast Activation In vitro

Camilo Mora-Navarro[†,‡], Andreea Badileanu[†,‡], Ana M. Gracioso Martins [†,‡], Emily W. Ozpinar[†,‡], Lewis Gaffney[†,‡], Ian Huntress[§], Erin Harrell[§], Jeffrey R. Enders[⊥], Xinxia Peng[§,#], Ryan C. Branski[ǁ], and Donald O. Freytes [†,‡]*

Equation S1 $\boldsymbol{Normalized\ absorbance_{405\ nm}} = \frac{\boldsymbol{A_x - A_0}}{\boldsymbol{A_{Max.} - A_0}}$

A is the absorbance value read at that time point. Meanwhile, A_x refers to any time point, A_0 the minimum absorbance measured. $A_{MAX.}$ is the maximum absorbance read.

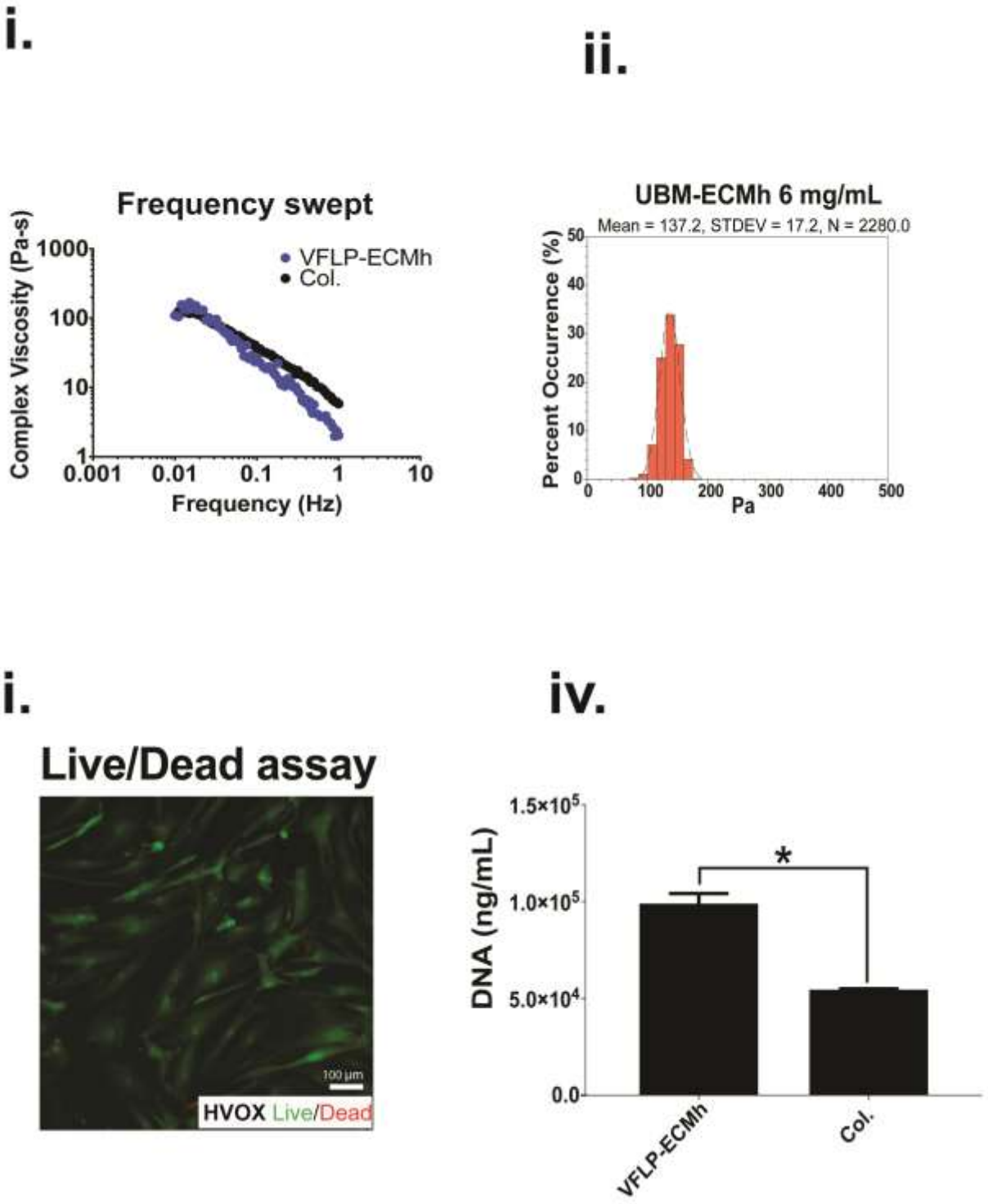
Equation S2 $|\eta^*| = kf^n$;

Equation S3 $Log|\eta^*| = nLogf + Logk$ [1]

Proteomics Method S1

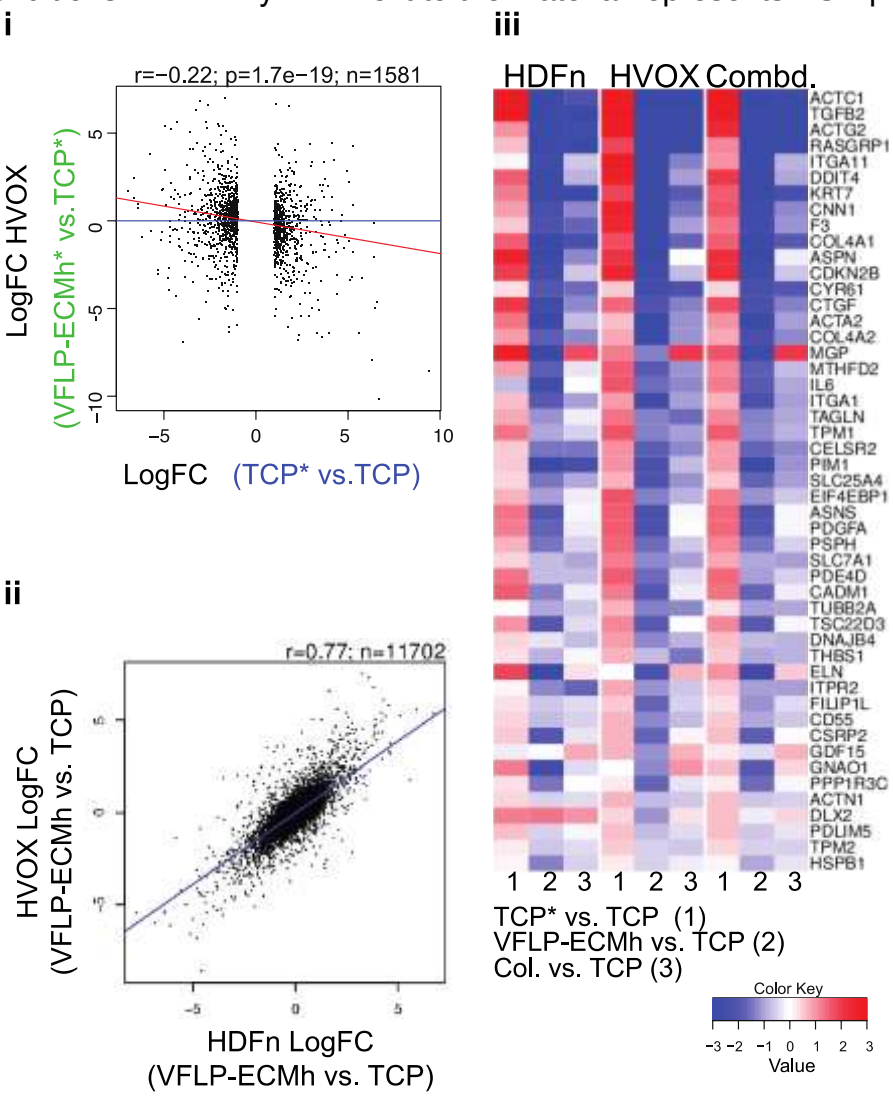
The digested ECM and resulting suspensions were vortexed and sonicated for 1 min at 20% amplitude on a Fisher Scientific Sonic Dismembrator, Model 120. Samples were spun down at 12,000 x g-force for 15 minutes. Protein quantitation was performed on the supernatant of these spins using a Pierce bicinchoninic acid (BCA) protein assay kit. The amount of protein in each sample was adjusted using 5% sodium deoxycolate (SDC) solution in 50 mM ammonium bicarbonate such that the final amount of protein was 20 µg in 200 µL (i.e., 0.1 µg μL^{-1}). Dithiothreitol (DTT) was added to each sample to make a final concentration of 5 mM and then incubated at 60 °C for 30 minutes in order to reduce disulfide bonds. Following the reduction, samples were cooled to room temperature and iodoacetamide (IAM) was added to a final concentration of 15 mM and incubated in the dark for 20 minutes at room temperature. Samples were then washed twice with 50mM ammonium bicarbonate buffer containing 8 M urea using Sartorius Vivacon 500 spin filters with a 30 kDa molecular cutoff weight spun at 12,000 x g-force. Eluent from this step was discarded (proteins remain above the spin filter). Samples were then washed twice with 50 mM ammonium bicarbonate using the same conditions as previously; the eluent was discarded. The waste collection tube below the spin filter was replaced with a fresh tube. Tryptic digestion was achieved by hydrating lyophilized trypsin to a stock solution of 0.1 µg μL^{-1} with 0.01 %v/v acetic acid in water. The trypsin solution was added to the protein mixture (i.e. 20 µg protein) in a 1:50 ratio (~0.4 µg trypsin), and then incubated at 37 °C for 4 hours with shaking. Samples were then spun at 12,000 x g-force. At this stage, proteins had been digested into lower molecular weight peptides which could pass through the filter into the collection tube. Samples were next acidified with 6 M HCl to a final concentration of 250 mM (pH ≤ 3).

Figure S1



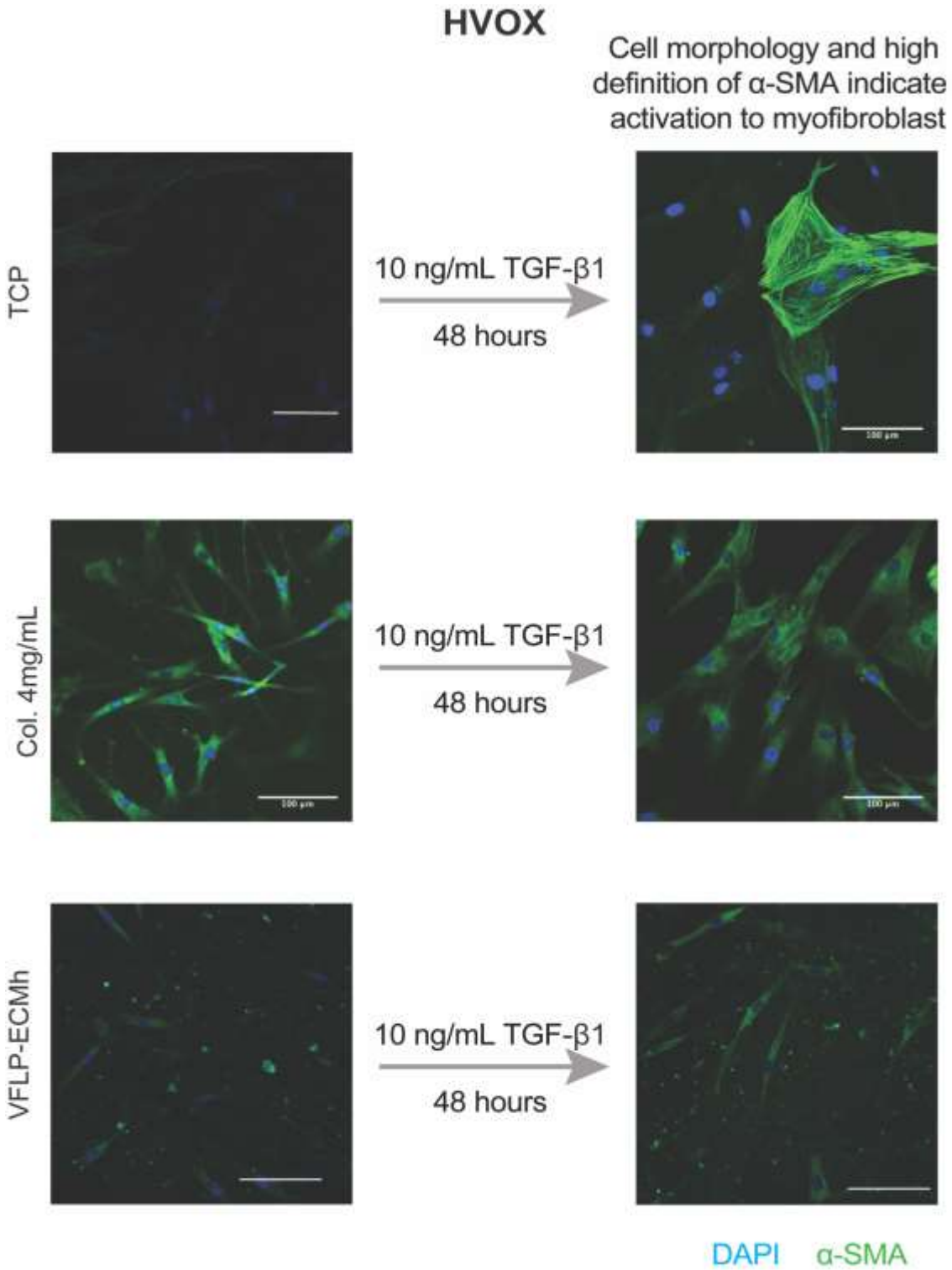
i. Complex viscosity behavior of VFLP-ECMh compared to Collagen Type I at 6 mg/mL. ii. Local surface modulus of the UBM-ECMh. iii. Live/Dead assay staining for HVOX growing on VFLP-ECMh: Calcein AM (live signal, green), ethidium homodimer-1 (dead signal, red). iv. dsDNA quantification for HVOX cultured on VFLP-ECMh or Col.

Figure S2 i) Scatterplot comparing gene expression changes in HVOX cells cultured on VFLP-ECMh* and TCP* vs. cells cultured on TCP* and TCP **ii) <u>Scatterplot of global gene expression</u>** changes induced in HVOX vs. HDFn by the VFLP-ECMh. **iii) <u>Heat map</u>** with the same annotated TGF-β1 targeted genes used in Figure 3A. to evaluate similarities in gene expression between HDFn and HVOX upon the three different treatment conditions. The "*" symbol next to the material represents TGF-β1 treatment.



i) X-axis: log2 fold changes in gene expressions in HVOX grown on TCP* compared to cells on TCP only. Y-axis: log2 fold changes in gene expressions in cells cultured on VFLP-ECMh* compared to cells on TCP*. Only the genes that showed a significant difference between TCP* and TCP were considered for the plot. The blue line reference indicates no gene expression difference between cells in VFLP-ECMh* and cells on TCP*. The red line represents a regression line across the genes shown. r and p: test of association between these paired log2 fold changes using Pearson's product moment correlation coefficient implemented in R's function 'cor.test'. ii) the scatter plot represents a comparable shift in the genes upon culture on VFLP-ECMh in reference to TCP for HVOX vs. HDFn. iii) Heatmap shows a subset of genes manually selected from those genes that: 1) were significantly upregulated in HVOX grown on TCP*; 2) exhibited significant difference between VFLP-ECMh* vs. TCP* (Green); 3) predicted TGF-β1 targets; 4) were downregulated by VFLP-ECMh on both HVOX and human dermal fibroblast HDFn

Figure S3 Fibroblast stimulation to myofibroblast using α-SMA as a marker.



The images were processed as described in the method section.

Figure S4 Gene association (GeneMania) TGF-β1 physical interaction report

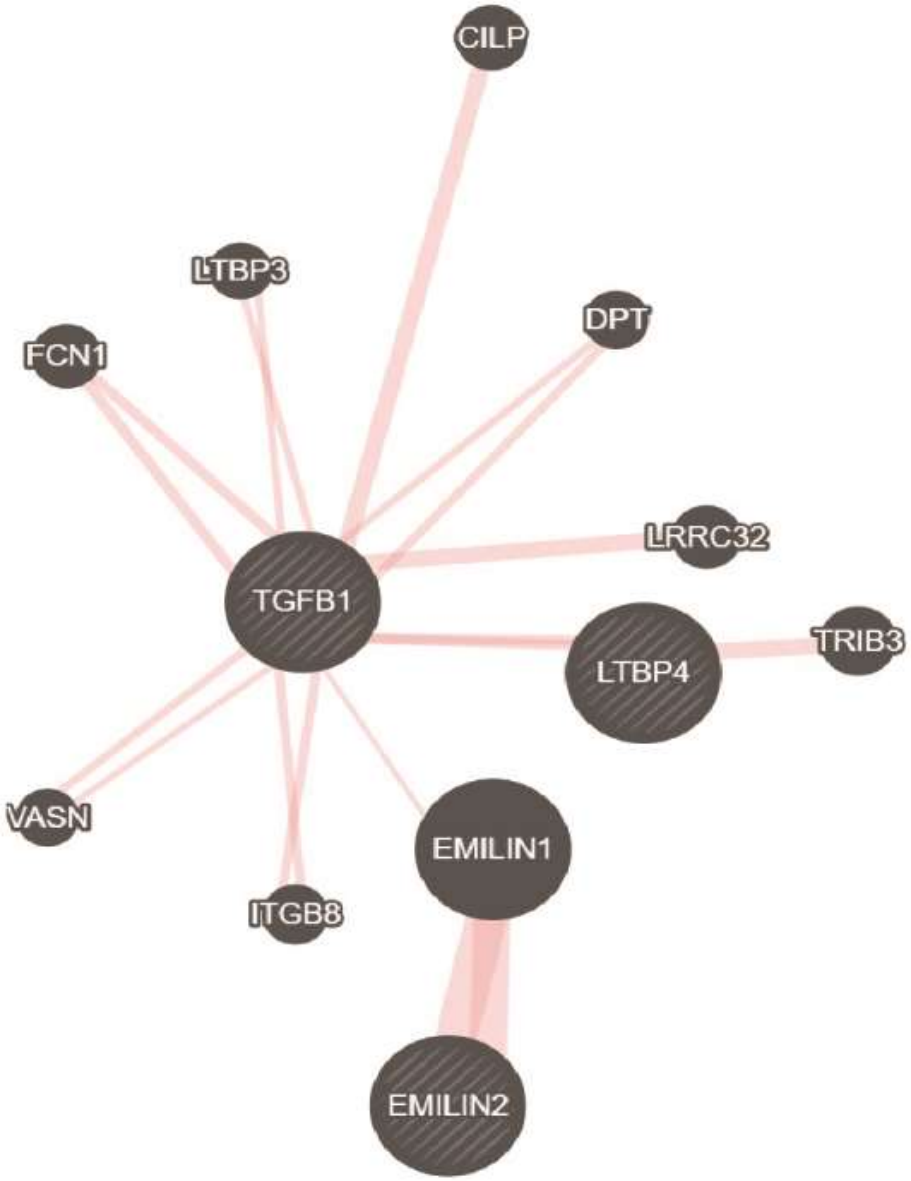


Table S1 List of primer sequences used for real-time quantitative polymerase chain reaction RT-qPCR.

ACTA2	Forward	GTGTTGCCCCTGAAGAGCAT
	Reverse	GCTGGGACATTGAAAGTCTCA
COL1A1	Forward	GTGCGATGACGTGATCTGTGA
	Reverse	CGGTGGTTTCTTGGTCGGT
GAPDH	Forward	AAGGTGAAGGTCGGAGTCAAC
	Reverse	GGGGTCATTGATGGCAACAATA

Table S2 Analysis of complex viscosity and comparison with other hydrogels injected in the Vocal Folds

Material	***k***	***n***	***r²***	***Frequency range (Hz)***	***Reference***
VFLP-ECMh	2.565	- 0.982	0.984	0.01-1	Figure SI.i
Col.	7.069	- 0.703	0.991	0.01-1	Figure SI i
UBM-ECMh 6 mg/mL	5.69	-0.955	0.999	0.01-15	[2]
Cymetra®	19.9	-0.778	0.972	0.01-100	[2]
Zyderm™	12	-0.860	0.977	0.01-100	[2]
Hyaluronic acid-DTPH	3.19	−0.744	0.974	0.01–100	[2]

Values were calculated using the data from the plot shown in Figure S1 and by applying the data to Equation S2 and Equation S3.

References:

1. Chan, R. W.; Titze, I. R., Viscosities of Implantable Biomaterials in Vocal Fold Augmentation Surgery. *The Laryngoscope* **1998,** *108* (5), 725-731. DOI: 10.1097/00005537-199805000-00019.
2. Freytes, D. O.; Martin, J.; Velankar, S. S.; Lee, A. S.; Badylak, S. F., Preparation and rheological characterization of a gel form of the porcine urinary bladder matrix. *Biomaterials* **2008,** *29* (11), 1630-7. DOI: 10.1016/j.biomaterials.2007.12.014.