Online Data Supplement

Title

LRP1 Regulation of Pulmonary Function: Follow-up of Human GWAS in Mouse.

Authors

[1]Cody E. Nichols, [2,3]John S. House, [1]Huiling Li, [4]James M. Ward, [5]Annah Wyss, [6]Jason G. Williams, [6]Leesa J. Deterding, [1]Jennifer A. Bradbury, [1]Laura Miller, [1]Darryl C. Zeldin, [1,5]Stephanie J. London

[1]Immunity, Inflammation, and Disease Laboratory, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina, United States

[2]Bioinformatics Research Center, North Carolina State University, Raleigh, North Carolina, United States

[3]Center for Human Health and the Environment, North Carolina State University, Raleigh, North Carolina, United States

[4]Integrative Bioinformatics Support Group, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina, United States

[5]Epidemiology Branch, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina, United States

[6]Mass Spectrometry Research and Support Group, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina, United States

Corresponding Author: Stephanie J. London, NIEHS, 111 TW Alexander Drive, PO Box 12233,

MD A3-05, Research Triangle Park, NC 27709, Email london2@niehs.nih.gov , phone 984-287-3688, fax (301) 480-3290

Supplemental Material and Methods

Human GWAS of LRP1 SNPs in relation to pulmonary function traits (FEV1, FVC and FEV1/FVC)

Two SNPs in *LRP1* were associated with FEV1/FVC in an earlier GWAS meta-analysis of 48,201 individuals of European ancestry (1): rs7968719 and rs11172113. More recently, a larger multi-ethnic meta-analysis from the Cohorts for Heart and Aging Research in Genomic Epidemiology (CHARGE) consortium evaluated all three pulmonary function traits (FEV1, FVC, and FEV1/FVC) in 90,715 individuals (2). Using the complete meta-analysis results files from this CHARGE multiethnic meta-analysis (publicly available in dbGAP under accession number phs000930) we created locus zoom plots of *LRP1* variants in relation to FEV_1, FVC and FEV_1/FVC using http://locuszoom.sph.umich.edu/ web-based software with a +/- 500kb flanking region and linkage disequilibrium (LD) based on hg19/1000 Genomes Mar 2012 EUR. In each locus zoom plot the most statistically significant SNP serves as the sentinel SNP.

Expression quantitative trait analysis of sentinel GWAS SNPs in LRP1

To investigate whether the genome wide significant GWAS SNPs in *LRP1* (rs7968719 and rs11172113) are associated with gene expression in cis (eQTLs), we searched for ENSG00000123384 (the Ensembl ID for *LRP1*) among significant cis-eQTL results from the GTEx database available for 48 tissues with more than 70 samples (median=166), including lung (release 7, downloaded on 3 January 2019, https://www.gtexportal.org/home/). Details of the GTEx data and methods are available online at https://www.gtexportal.org/home/documentationPage. We also examined whether these SNPs

were cis-eQTLs in blood in two larger databases of European ancestry individuals: Westra et al (N=5,311) (3) and BIOS (N=2,116) (4, 5).

Pulmonary Function Assessment

Knockout and control animals between 9-12 weeks of age underwent pulmonary function analysis using the flexiVent™ Legacy system or flexiVent™ FX2 system (SCIREQ, Inc, Montreal, Canada) as previously described (6). In this terminal procedure, urethane-anesthetized mice (1.5 g/kg, *i.p.*) were tracheotomized and injected with pancuronium bromide (0.8 mg/kg, *i.p.*) to preventing autonomous breathing. Mice were then connected to a computer-controlled ventilation device that performed pulmonary function perturbations at baseline and following methacholine (MCH) administration. For LPS experiments, male mice were exposed to 50 µg bacterial LPS (*Escherichia coli* O111:B4; L2630; Sigma-Aldrich; St. Louis, MO) in sterile saline (S8776, Sigma-Aldrich) via oropharyngeal aspiration (OPA), and four hours later analyses were conducted. For HDM experiments, male mice were sensitized with 10 µg HDME (*Dermatophagides pteronyssinus*, Greer XPB82D3A2.5, Lot number 329112: 6,170 endotoxin units/mL) in sterile saline via OPA on days 1 and 8, followed by challenge on days 15, 16, and 17 with 2 µg HDME, and measurements were collected on day 18.

Immunofluorescence

Pulmonary tissue was sampled from control and knockout animals and assessed for LRP1 expression using immunofluorescence. Paraformaldehyde fixed tissue mounted on charged microscope slides (Fisher Scientific, Pittsburgh, PA) was incubated overnight at 4°C with mouse anti-LRP1 (ab28320; Abcam, Cambridge, United Kingdom), Anti-CD31 antibody (ab28364,

Anti-EpCAM (ab71916, Abcam), and rat anti-alpha-smooth muscle actin (ab5694; Abcam). Following primary antibody incubation, slides were incubated in goat anti-mouse (ab6563; Abcam) and goat anti-rat (ab150081; Abcam) antibodies. Images were captured using a confocal microscope (ZEISS, Oberkochen, Germany) and colocalization was assessed using Zen Black (ZEISS, Oberkochen, Germany). Colocalization was determined from three nonoverlapping regions of each cross section and represented as a percentage of the total signal collected with each filter. Representative negative control and specific cell marker images are available in Figure E2.

Bronchoalveolar Lavage Fluid Proteomic Analyses

Samples were digested overnight with trypsin (1 µg/µL) at 37°C. The digests were then stored at -80°C for subsequent MS analysis. Protein digests were analyzed by LC/MS on a Q Exactive Plus mass spectrometer (ThermoFisher Scientific,Waltham MA) interfaced with an M-Class nanoAcquity UPLC system (Waters Corporation, Milford MA) equipped with a 75 µm x 150 mm BEH dC18 column (1.8 µm particle, Waters Corporation, Milford MA) and a C18 trapping column (180 µm × 20 mm) with 5 µm particle size at a flow rate of 400 nL/min. The trapping column was positioned in-line of the analytical column and upstream of a micro-tee union which was used both as a vent for trapping and as a liquid junction. Trapping was performed using the initial solvent composition. 5 µL of digested sample was injected onto the column. Peptides were eluted by using a linear gradient from 99% solvent A (0.1% formic acid in water (v/v)) and 1% solvent B (0.1% formic acid in acetonitrile (v/v)) to 40% solvent B over 70 minutes.

For the mass spectrometry, a top-ten data dependent acquisition method was employed with a dynamic exclusion time of 15 seconds and an exclusion of +1 charge states. The mass spectrometer was equipped with a nanoflex source with a stainless-steel needle and was used in the positive ion mode. Instrument parameters were as follows: sheath gas, 0; auxiliary gas, 0; sweep gas, 0; spray voltage, 2.7 kV; capillary temperature, 275 °C; S-lens, 60; scan range (m/z) of 375 to 1500; 1.6 m/z isolation window; resolution: 70,000 (MS), 17,500 (MS/MS); automated gain control (AGC), 3 × 10e6 ions (MS), 5 x 10e4 (MS/MS); and a maximum IT of 100 ms (MS), 50 ms (MS/MS). Mass calibration was performed before data acquisition using the Pierce LTQ Velos Positive Ion Calibration mixture (ThermoFisher Scientific, Waltham MA). The LC/MS data were analyzed using Proteome Discoverer software (ThermoFisher Scientific, Waltham MA). Each sample were acquired in triplicate and the results were searched against the RefSeq mouse protein database.

Statistical Analysis

For assessment of genotype differences in pulmonary function parameters at baseline and after MCH administration we used a Student's t-test. Values were reported as mean ± standard error of the mean (SEM) and considered significant if the p-value for difference by genotype was less than 0.05.

Peptide-level abundances were analyzed in R. The log2-transformed abundances were median-normalized and evaluated for technical outliers using MA-plots with an outlier threshold of 2x median absolute deviation factor applied within each technical triplicate. Technical replicates were combined using the mean log2 normalized abundance, then statistical comparisons

performed using limma (7, 8) moderated t-test, comparing *Lrp1*$^{-/-}$ to *Lrp1*$^{+/+}$. The proteomic approach employed does not provide absolute quantification; comparisons of a protein across groups are valid but comparisons of levels of different proteins are not. Hence, we present fold changes but not cannot provide absolute levels. Statistically significant associations were defined using Benjamini Hochberg adjusted P-value=0.05 and absolute fold change of 1.5. Alternatively, peptides were combined to protein level using the sum of the exponentiated normalized abundance. The protein abundances were then log2-transformed prior to statistical comparison using limma as described for peptides.

Supplemental Figure Legends

Figure E1. Baseline pulmonary function and airway responsiveness unaffected by SM-Cre recombinase allele. (A) Assessment of baseline pulmonary function in SM-Cre^{+} and SM-Cre^{-} animals. (B) Assessment of airway responsiveness to methacholine (MCH) in SM-Cre^{+} and SM-Cre^{-} animals. Means and SEMs are plotted (N ≥ 6/genotype).

Figure E2. Immunofluorescence controls for LRP1. Representative immunofluorescent images for DAPI (Blue) and LRP1 (Cy5-White). These sections were treated with either secondary antibodies only (goat anti-mouse & goat anti-rat), or with both anti-LRP1 and secondaries. Scale bar = 20 μm.

Figure E3. LRP1 expression in endothelial epithelial cells is not affected in *Lrp1*$^{-/-}$ mice. Representative immunofluorescent images of *Lrp1*$^{+/+}$ and *Lrp1*$^{-/-}$ mice. Lung sections from *Lrp1*$^{+/+}$ and *Lrp1*$^{-/-}$ mice were incubated with anti-LRP1 and either anti-CD31 (columns 1&2) or anti-EpCAM (columns 3&4) followed by appropriate secondary antibodies. (A) DAPI (blue) and LRP1 immunofluorescence (IF; Cy5). (B) DAPI (blue) and either CD31 IF (Alexa488; columns 1&2) or EpCAM IF (Alexa488; columns 3&4). (C) Merge of DAPI (blue), LRP1 (Cy5) and either CD31 (Alexa488; columns 1&2) or EpCAM (Alexa488; columns 3&4). (D) DAPI (blue) and co-localized LRP1 with either CD31 (columns 1&2) or EpCAM (columns 3&4) falsely colored yellow. CD31/EpCAM expressed without LRP1 in green. (E) Quantification of co-localization signal in *Lrp1*$^{+/+}$ and *Lrp1*$^{-/-}$ animals for LRP1 and CD31; n = 3/genotype. (F) Quantification of co-localization signal in *Lrp1*$^{+/+}$ and *Lrp1*$^{-/-}$ animals for LRP1 and EpCam. n = 3/genotype; scale bars = 20μm.

References:

1. Soler Artigas M, Loth DW, Wain LV, Gharib SA, Obeidat M, Tang W, Zhai G, Zhao JH, Smith AV, Huffman JE, et al. Genome-wide association and large-scale follow up identifies 16 new loci influencing lung function. *Nat Genet* 2011;43(11):1082-1090.
2. Wyss AB, Sofer T, Lee MK, Terzikhan N, Nguyen JN, Lahousse L, Latourelle JC, Smith AV, Bartz TM, Feitosa MF, et al. Multiethnic meta-analysis identifies ancestry-specific and cross-ancestry loci for pulmonary function. *Nat Commun* 2018;9(1):2976.
3. Westra HJ, Peters MJ, Esko T, Yaghootkar H, Schurmann C, Kettunen J, Christiansen MW, Fairfax BP, Schramm K, Powell JE, et al. Systematic identification of trans eqtls as putative drivers of known disease associations. *Nat Genet* 2013;45(10):1238-1243.
4. Bonder MJ, Luijk R, Zhernakova DV, Moed M, Deelen P, Vermaat M, van Iterson M, van Dijk F, van Galen M, Bot J, et al. Disease variants alter transcription factor levels and methylation of their binding sites. *Nat Genet* 2017;49(1):131-138.
5. Zhernakova DV, Deelen P, Vermaat M, van Iterson M, van Galen M, Arindrarto W, van 't Hof P, Mei H, van Dijk F, Westra HJ, et al. Identification of context-dependent expression quantitative trait loci in whole blood. *Nat Genet* 2017;49(1):139-145.
6. House JS, Li H, DeGraff LM, Flake G, Zeldin DC, London SJ. Genetic variation in htr4 and lung function: Gwas follow-up in mouse. *FASEB J* 2015;29(1):323-335.
7. Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge YC, Gentry J, et al. Bioconductor: Open software development for computational biology and bioinformatics. *Genome Biology* 2004;5(10).
8. Smyth GKG, R.; Carey, V.; Dudoit, S.; Irizarry, R.; Huber W. Limma: Linear models for microarray data. . Springer; 2005.

Table E1**.** Provocative Concentration of Methacholine Aerosol at which a 200% Increase Over Baseline was Observed in Naïve Mice.

	Lrp1$^{+/+}$	*Lrp1*$^{-/-}$		
	Average±SEM	Average±SEM	% Control	P-Value
Resistance	8.95±1.59	4.17±1.17	46.55	0.035
Compliance	29.96±1.71	27.07±1.69	90.35	0.244
Elastance	33.86±7.46	24.39±2.71	74.97	0.275
Airway Resistance	8.69±1.17	5.16±0.83	59.41	0.021
Tissue Resistance	14.28±1.75	8.26±2.24	57.84	0.041
Tissue Elastance	47.32±2.95	51.52±5.53	108.88	0.508

Abbreviations: Lrp1 =Low Density Lipoprotein receptor related protein 1, SEM = Standard error of the mean.

Table E2. Provocative Concentration of Methacholine Aerosol at which a 200% Increase Over Baseline was Observed in Lipopolysaccharide Exposed Mice.

	Lrp1$^{+/+}$	*Lrp1*$^{-/-}$		
	Average±SEM	Average±SEM	% Control	P-Value
Resistance	7.15±1.21	6.30±0.72	88.11	0.537
Compliance	30.47±2.89	29.04±1.50	95.31	0.650
Elastance	16.76±9.57	21.55±2.30	128.58	0.607
Airway Resistance	4.82±0.75	6.26±0.69	129.88	0.169
Tissue Resistance	14.20±2.94	11.97±1.23	84.30	0.468
Tissue Elastance	44.62±9.66	34.04±3.94	76.29	0.294

Abbreviations: Lrp1 =Low Density Lipoprotein receptor related protein 1, SEM = Standard error of the mean.

Table E3**.** Provocative Concentration of Methacholine Aerosol at which a 200% Increase Over Baseline was Observed in House Dust Mite Extract Exposed Mice.

	Lrp1$^{+/+}$	*Lrp1*$^{-/-}$		
	Average±SEM	Average±SEM	% Change	P-Value
Resistance	37.00±8.14	25.56±4.75	69.10	0.271
Compliance	119.80±39.36	75.07±7.74	62.66	0.335
Elastance	124.53±43.96	64.82±13.56	52.0543	0.258
Airway Resistance	56.04±13.33	52.75±29.28	94.14	0.913
Tissue Resistance	66.44±20.05	34.86±7.72	52.46	0.197
Tissue Elastance	106.96±28.39	117.36±33.24	109.73	0.885

Abbreviations: Lrp1 =Low Density Lipoprotein receptor related protein 1, SEM = Standard error of the mean.

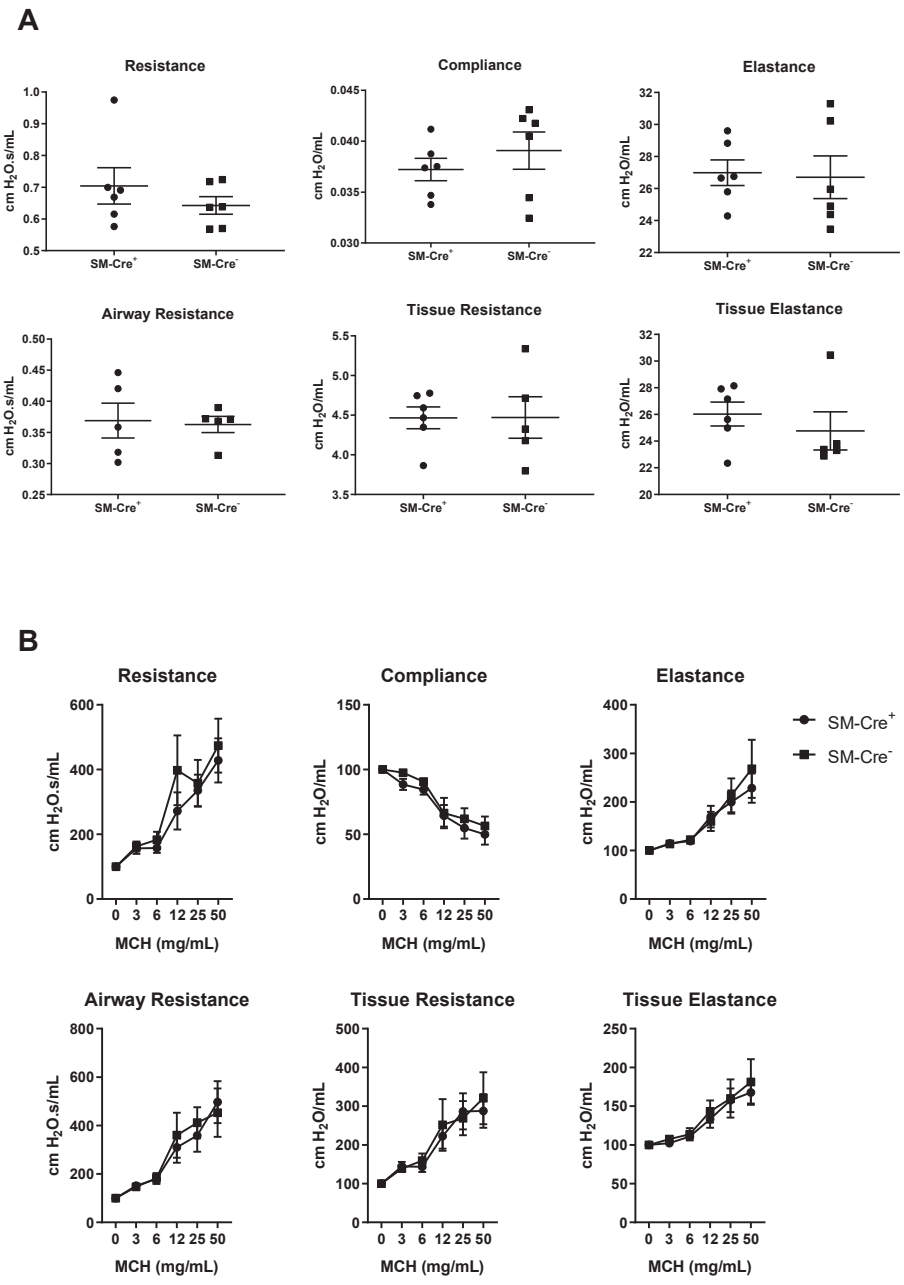

A
Resistance
cm H2O.s/mL
SM-Cre+
SM-Cre-
Compliance
cm H2O/mL
Elastance
cm H2O/mL
Airway Resistance
cm H2O.s/mL
Tissue Resistance
cm H2O/mL
Tissue Elastance
cm H2O/mL
B
Resistance
cm H2O.s/mL
MCH (mg/mL)
Compliance
cm H2O/mL
Elastance
cm H2O/mL
SM-Cre+
SM-Cre-
Airway Resistance
Tissue Resistance
Tissue Elastance

Figure E2

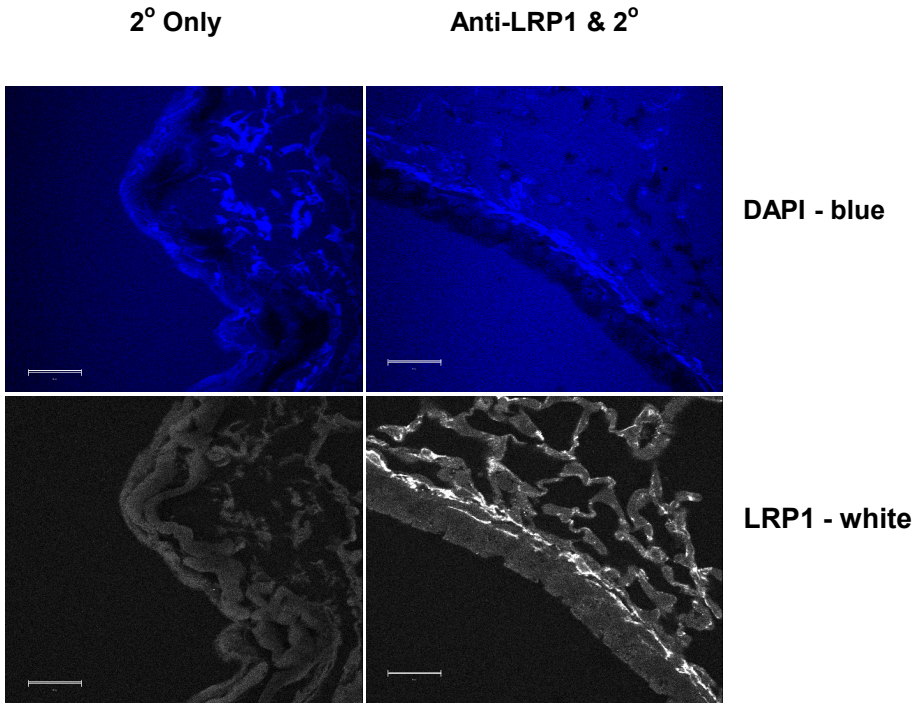
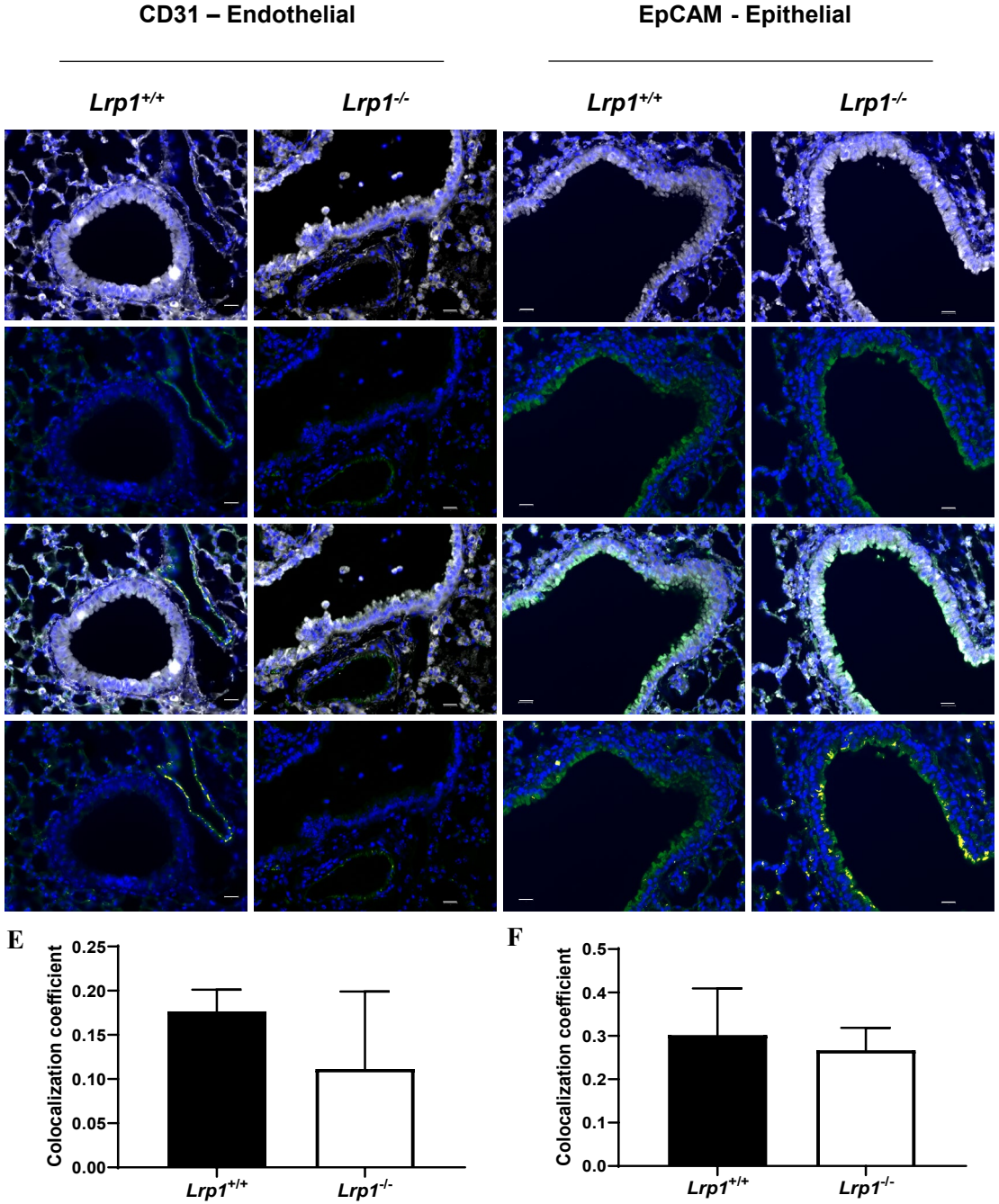


Figure E3


CD31 – Endothelial
EpCAM - Epithelial
Lrp1+/+
Lrp1-/-
Lrp1+/+
Lrp1-/-
(A)
DAPI - blue
LRP1 - white
(B)
DAPI - blue
CD31/EpCAM - green
(C)
DAPI – blue
LRP1 - white
CD31/EpCAM - green
(D)
DAPI – blue
LRP1+CD31/EpCAM - yellow
CD31/EpCAM - green
E
Colocalization coefficient
0.25
0.20
0.15
0.10
0.05
0.00
Lrp1+/+
Lrp1-/-
F
Colocalization coefficient
0.5
0.4
0.3
0.2
0.1
0.0
Lrp1+/+
Lrp1-/-